Faculty of Pharmaceutical Sciences

HUMAN BIOMONITORING OF MYCOTOXIN EXPOSURE THROUGH BIOMARKER ANALYSIS

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General introduction

CHAPTER 1

GENERAL INTRODUCTION TO MYCOTOXINS

1.1 DEFINITION OF MYCOTOXINS

Mycotoxins are secondary metabolites produced by fungi. These naturally occurring toxins are not associated with fungal growth, but are a consequence of the fungus responding to signals from the environment (Moss, 1991). Mycotoxins are produced under specific conditions of moisture and temperature and are generally associated with diseased or mouldy crops. Not all fungi can produce mycotoxins, and even those with the ability to produce mycotoxins may not produce them all the time. Fungi can contaminate crops with mycotoxins on the field (*pre-harvest*) or during transport, storage or processing (*post-harvest*) with *Aspergillus*, *Penicillium* and *Fusarium* as main producers. Mycotoxins are physically and chemically very stable and tend to survive storage and food processing even at extreme temperatures. Nevertheless, those that do occur in food have great impact on the health of humans and can cause significant economic losses in terms of plants and livestock.

Mycotoxins form a worldwide problem as they are present in more than 25 % of the worldwide crops (CAST, 2003). Mycotoxins occur in different food commodities such as cereals, nuts, (dried) fruit, coffee, spices, seeds or beans and are responsible for a diverse range of toxic effects because of their different chemical structures. While exposure through ingestion of contaminated food is numerously reported, inhalation and skin contact can also be a direct route of exposure (Hendry and Cole, 1993; Pitt et al., 2000). Furthermore, people can also be exposed to mycotoxins by the consumption of animal derived products such as meat, eggs or milk (carry-over). Exposure to toxic fungal metabolites causes diseases collectively called mycotoxicoses which can be categorised as acute or chronic (Forgacs, 1962). Acute toxicity generally has a rapid onset and an obvious toxic response. It requires

high amounts of toxin present in food being consumed and such incidents are usually restricted to the less developed parts of the world where resources for control are limited (Bennett and Klich, 2003). Chronic effects are caused by the exposure of a low level of toxin in the body over a long period of time and can affect the long-term health of the population (James, 1985). Mycotoxins can be carcinogenic, genotoxic or may target kidney, liver or immune system (see chapter 2). The magnitude of effect is influenced by many factors such as dose, duration of exposure, age and health status of the exposed individual (Bennett and Klich, 2003). The toxicity and mode of action of mycotoxins is complex due to the conversion into metabolites which can lead to bioactivation or inactivation (see chapter 2). Since fungal species are able to produce more than one mycotoxin there could be an additive, synergetic or antagonistic effect (Speijers and Speijers, 2004).

Mycotoxin exposure differs around the world, depending on the climate. In general, in developing countries where poor methods of food handling and storage are common and where only a few or no regulations exist, mycotoxin exposure is more likely to occur (Bennett and Klich, 2003). However, even in developed countries, specific subgroups may be vulnerable to mycotoxin exposure. As climate change affects environmental factors, this could have an important impact on mycotoxin contamination (Miraglia et al., 2009). Methods for controlling mycotoxins are largely preventive. None of these methods has solved the problem, because mycotoxin formation is often unavoidable. Many efforts to address the mycotoxin problem simply involve the elimination of mycotoxin contaminated commodities from the food supply through government screening and regulatory programs. Still the economic losses, the risk for animal and human health are underestimated.

1.2 MAJOR MYCOTOXINS

The term mycotoxin was coined in 1962 after the death of 100 000 turkeys in England. When this mysterious turkey X disease was linked to a peanut meal contaminated with secondary metabolites from *A. flavus* (aflatoxins), it sensitised scientists to the possibility

that other unknown mold metabolites might be deadly (Blout, 1961; Forgacs, 1962). For this reason, the period between 1960 and 1975 has been termed the mycotoxin gold rush (Maggon et al., 1977). Many scientists joined the well-funded search for these toxigenic agents. The turkey X disease was not the first disease caused by exposure to mycotoxins. Ergotism was common in the Middle Ages. It is also known as Saint Anthony's Fire and was caused by the ingestion of ergot alkaloids produced by *Claviceps* species. Ergotism can be roughly divided in two forms namely the gangrenous form that affects the blood supply to the extremities while the convulsive form affects the central nervous system (Bennett and Bentley, 1999). Another example of a historical mycotoxicosis is Alimentary Toxic Aleukia (ATA). ATA affected a large part of the Russian population during World War II with symptoms including inflammation of the skin, vomiting and central nervous system disorders. ATA was caused by food contaminated with trichothecenes (Matossian, 1981). More recently, mycotoxins were linked to the sick-building syndrome. Poor ventilation, water damage and molds in indoor environments are responsible for irritation of the eyes and respiratory tract (Nielsen et al., 1999). Currently more than 400 mycotoxins are identified. Within the scope of this doctoral thesis the most abundant mycotoxins are described with emphasis on those with available (potential) biomarkers of exposure.

1.2.1 Aflatoxins

Aflatoxins are difuranocoumarin derivatives (figure 1.1.) produced by a polyketide pathway by many species of *Aspergillus* such as *A. flavus* and *A. parasiticus* (Peterson et al., 2001). Aflatoxins predominantly occur in hot and humid regions of the world and contaminate dietary staples including maize and groundnuts. Sometimes crops become contaminated with aflatoxins in the field before harvest, where it is usually associated with drought stress (Diener et al., 1987). Higher levels of fungal growth and toxin production often occur during storage where moisture content of the substrate and the relative humidity of the surroundings are the most important variables (Wilson and Payne, 1994). The four major aflatoxins are called aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2), based on their fluorescence under UV light (blue or green), with AFB_1 being the

most toxic and carcinogenic within this group (Squire, 1981). Furthermore, AFB_1 can be hydroxylated to aflatoxin M_1 (AFM_1), which is a frequent metabolite in milk of exposed lactating animals, including in human breast milk after maternal exposure to dietary AFB_1 (Wild and Gong, 2010).

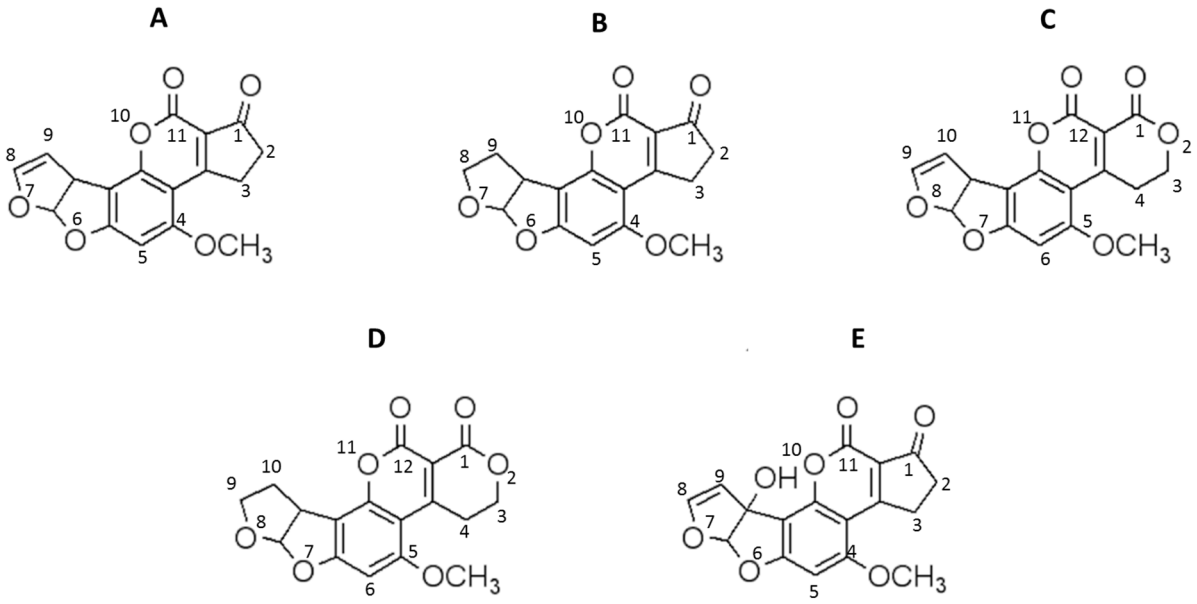


Figure 1.1. Structure of aflatoxin B_1 (A), aflatoxin B_2 (B), aflatoxin G_1 (C), aflatoxin G_2 (D) and aflatoxin M_1 (E)

1.2.2 Citrinin

Citrinin (CIT) was first isolated from *P. citrinum* prior to World War II (Hetherington and Raistrick, 1931). Subsequently, it was identified in different species of *Penicillium* and *Aspergillus.* More recently, CIT has also been isolated from *Monascus ruber* and *M. purpureus,* industrial species used to produce red pigments (Blanc et al., 1995). CIT is associated with the yellow rice disease in Japan (Saito et al., 1971) and can be found in wheat, oats, rye, maize, barley and rice (Abramson et al., 2001). CIT was also detected in certain vegetarian foods colored with *Monascus* pigments (Chu, 1991). CIT has structural similarities (figure 1.2.) with ochratoxin A (OTA) that could explain the similar toxicological effects and their additive mechanisms (Bennett and Klich, 2003; Chang et al., 2011).

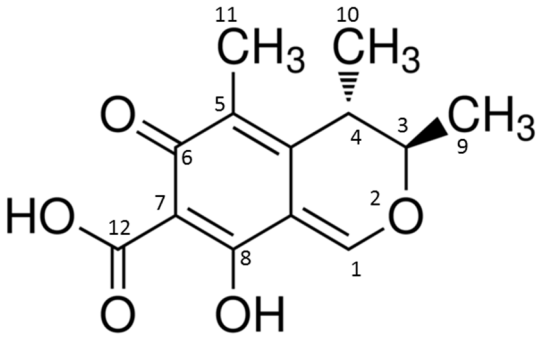


Figure 1.2. Structure of citrinin

1.2.3 Fumonisins

Fumonisins are a family of mycotoxins produced by the fungi *F. verticillioides* and *F. proliferatum* (Rheeder et al., 2002). Fumonisins were first described and characterised in 1988 (Gelderblom et al., 1988). Fumonisins predominantly contaminate maize in hot and humid climates, and co-contamination with aflatoxins is reported. Unlike most known mycotoxins, which are soluble in organic solvents, fumonisins are hydrophilic (figure 1.3.). The fumonisin story raises the specter that there may be many other unknown toxic products of fungal metabolism that have not yet been discovered because of their hydrophilic nature. Fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) are most commonly detected in maize and FB_1 is recognised as the most toxic fumonisin (Bennett and Klich, 2003).

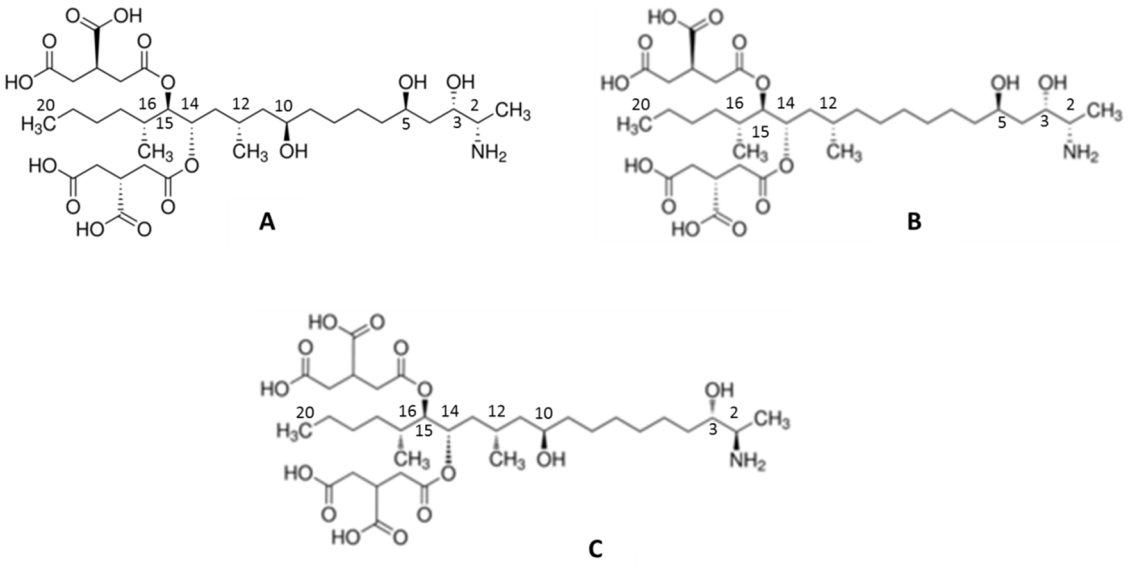


Figure 1.3. Structure of fumonisin B_1 (A), fumonisin B_2 (B) and fumonisin B_3 (C)

1.2.4 Ochratoxins

Members of the ochratoxin family (figure 1.4.) have been found as metabolites of many different species of *Aspergillus* and *Penicillium*. The ochratoxins are pentaketides made up of dihydro-isocoumarin linked to β-phenylalanine and consist of OTA, its methyl ester, its ethyl ester better known as ochratoxin C (OTC), ochratoxin B (OTB) and its methyl- and ethyl esters. OTA is the most toxic member in this group and was discovered as a metabolite of *A. ochraceus* in 1965 during a large screening of fungal metabolites that was designed specifically to identify new mycotoxins (Van der Merwe et al., 1965). In temperate regions, OTA is mainly produced by *P. verrucosum*, a common contaminant of barley (Pitt, 1987). The production of OTA during storage is the most important factor of OTA contamination in food. It has been found in barley, oats, rye, wheat, coffee beans and other plant products. There is also concern that OTA may be present in certain wines, especially those from grapes contaminated with *A. carbonarius* (Marquardt and Frohlich, 1992; Pitt, 2000).

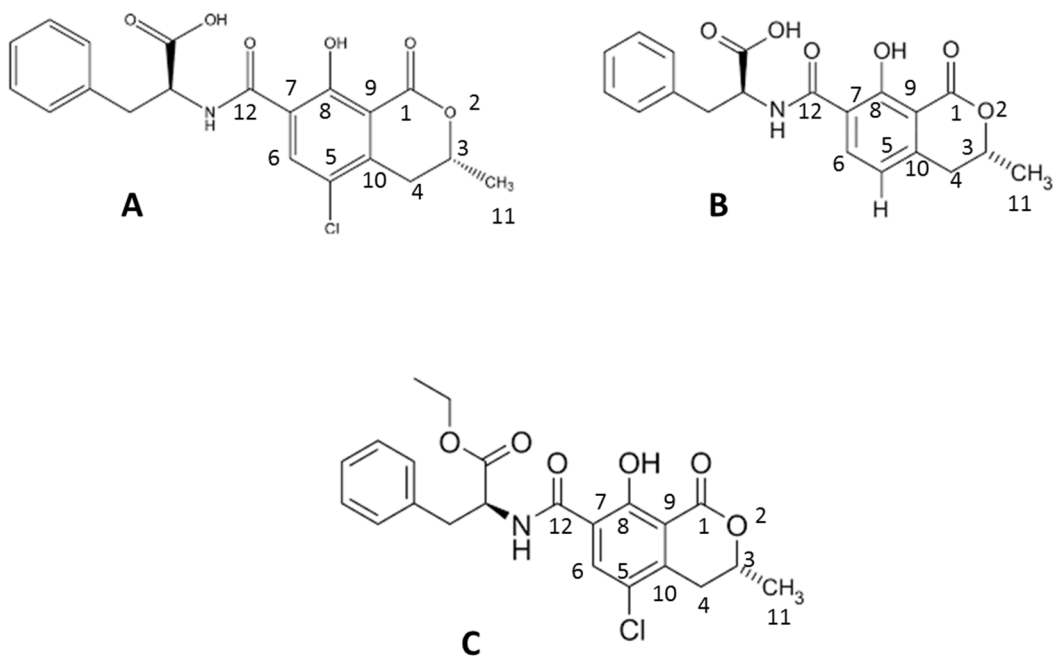


Figure 1.4. Structure of ochratoxin A (A), ochratoxin B (B) and ochratoxin C (C)

1.2.5 Trichothecenes

Trichothecenes are a family of mycotoxins that consists of more than 60 sesquiterpenoid metabolites produced by several *Fusarium* fungi that contaminate cereal crops throughout the world (Ueno, 1983). The term trichothecene is derived from trichothecin, which was one of the first members of the family identified. All trichothecenes contain a common 12,13-epoxytrichothene skeleton and an olefinic bond with various side chain substitutions (figure 1.5.). Trichothecenes are classified as macrocylic or nonmacrocyclic, depending on the presence of a macrocylic ester or an ester-ether bridge between C-4 and C-15 (Chu, 1998). The nonmacrocylic trichothecenes can be subclassified into two groups: type A, which have a hydrogen or ester type side chain at the C-8 position, and include T-2 toxin (T-2) and diacetoxyscirpenol (DAS), while the type B group contains a ketone and includes fusarenon X (FusX) and deoxynivalenol (DON). DON and T-2 are the best studied of the trichothecenes produced by *Fusarium* species. *F. graminearum* and *F. culmorum* infection of wheat and maize in more temperate regions causes significant economic loss in the form of head blight and contamination of DON and other trichothecene mycotoxins. DON, also known as

vomitoxin, is the most prevalent and is commonly found in barley, maize, rye, wheat and mixed feeds (Bennett and Klich, 2003). A survey within the European Union (EU) revealed that 44 % of food samples tested were contaminated with DON, demonstrating the frequent contamination (EFSA, 2013).

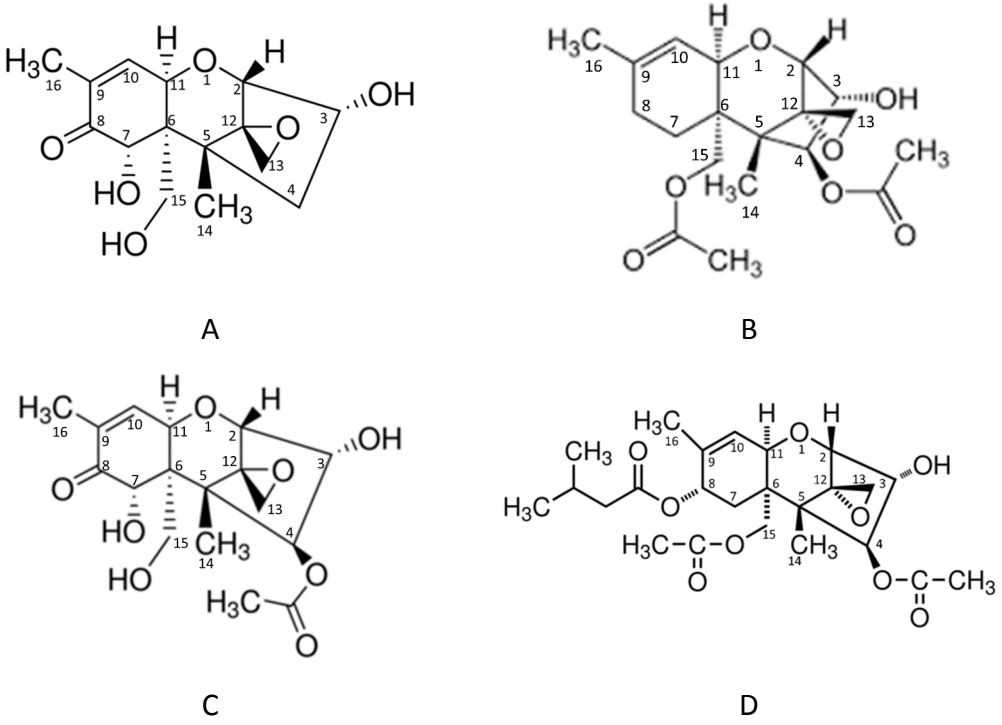


Figure 1.5. Structure of trichothecene mycotoxins deoxynivalenol (A), diacetoxyscirpenol (B), fusarenon X (C) and T-2 toxin (D)

1.2.6 Zearalenone

Zearalenone (ZEN) is a secondary metabolite of *F. graminearum* and *F. culmorum*. These species are regular contaminants of cereal crops worldwide (Hagler et al., 2001) and occur most of the time in temperate climates. ZEN and DON can contaminate crops simultaneously. The structure of ZEN is similar to 17β-estradiol, an important hormone produced by ovary of women, and is therefore better classified as a nonsteroidal estrogen or mycoestrogen (figure 1.6.). ZEN mainly contaminates maize, wheat, barley, oats and rye (Kuiper-Goodman et al., 1987).

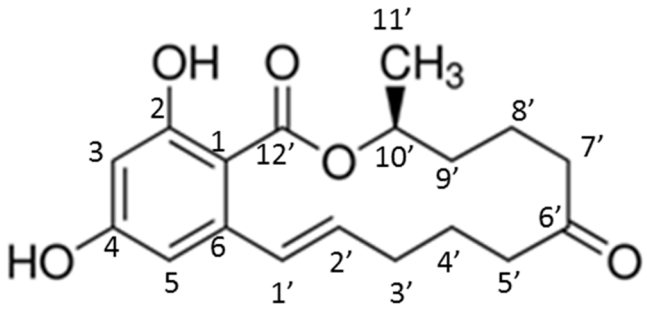


Figure 1.6. Structure of zearalenone

1.2.7 Modified mycotoxins

The term 'masked mycotoxins' has been introduced by Gareis et al. (1990) to describe zearalenone-14-glucoside (ZEN14Glc) since this glycoside cannot be detected during routine analysis, but is hydrolysed during digestion. In 2011, the International Life Science Institute (ILSI) has adapted the definition whereby masked mycotoxins are defined as mycotoxin derivatives that are undetectable by conventional analytical techniques because their structure has been changed in the plant. However, there are also other substances derived from mycotoxins, which are likewise not detectable in routine analysis, but which are primarily not produced by plants (Berthiller et al., 2013). In order to avoid misunderstanding the term modified mycotoxins has been introduced by Rychlik et al. (2014) and includes all potential mycotoxin derivatives. Table 1.1. gives an overview of this systematic definition of the modified mycotoxins. The emphasis of this definition has been placed on the processes whereby these modified toxins can be generated in order to encompass all possible forms. As some molecules can be generated in different ways, some compounds belong to more than one category (Rychlik et al., 2014).

According to this definition the term **'free or unmodified'** mycotoxins describes the basic mycotoxin structures formed as toxic secondary metabolites by various fungi in well-known biosynthetic pathways. Examples are OTA, AFB_1, FB_1, ZEN and DON. **Matrix-associated** mycotoxins are used for mycotoxins, which (1) form either complexes with matrix

compounds or are physically dissolved or trapped or (2) are covalently bound to matrix components or a combination of both effects. Examples for covalently bound forms of mycotoxins are fumonisins bound to starch or proteins (Seefelder et al., 2003; Shier, 2000). Recently also the covalent binding of OTA to polysaccharides via the carboxylic acid group of OTA during the roasting of coffee has been shown by Bittner et al. (2013). Also, DON-oligosaccharides have been described recently (Zachariasova et al., 2012).

Table 1.1. Systematic definition of modified mycotoxins (Rychlik et al., 2014)

Free or unmodified mycotoxins			
Matrix-associated mycotoxins	- Complexes, physically dissolved or trapped		
	- Covalently bound		
Modified mycotoxins	- Biologically modified	- Functionalised (Phase I metabolites)	
		- Conjugated by (Phase II metabolites)	- Plants - Animals - Fungi
		- Differently modified	
	- Chemically modified	- Thermally formed	
		- Non-thermally formed	

The term **'modified mycotoxins'** describes any modification of the basic chemical structure of mycotoxins either by chemical or biological modifications. **Biologically modified** mycotoxins include any functionalisation during phase I metabolism, for example AFB_1-exo-8,9-epoxide, which is the aflatoxin metabolite that reacts covalently with deoxyribonucleic acid (DNA) to form adducts responsible for toxic effects (see chapter 2). Furthermore, mycotoxin conjugates such as phase II metabolites are also defined as biologically modified. These include (1) conjugation reactions by plants such as the formation of deoxynivalenol-3-glucoside (DON3Glc) or ZEN14Glc, which are defined as masked mycotoxins (Berthiller et al., 2013); (2) conjugation reactions by animals such as the formation of deoxynivalenol-3-glucuronide (DON3GlcA) or deoxynivalenol-15-glucuronide (DON15GlcA) (Uhlig et al., 2013; Welsch and Humpf, 2012); and (3) conjugations by fungi as for example the formation of

zearalenone-14-sulphate (ZEN14S) (Plasencia and Mirocha, 1991). All other biological modifications are summarised under the term 'differently modified' and include for example deepoxy-deoxynivalenol (DOM-1) as an intestinal metabolite of DON, which is formed by the microbiota of animals and humans (Eriksen et al., 2003; Gratz et al., 2013).

Chemically modified mycotoxins are currently the largest group of modified mycotoxins and can be classified as 'thermally and non-thermally formed'. Thermal modifications occur during food and feed processing including baking, roasting, frying, or extruding. Thermal degradation products have been described for several mycotoxins such as fumonisins (Humpf and Voss, 2004; Seefelder et al., 2001), DON (Bretz et al., 2006), OTA (Cramer et al., 2008) and T-2 (Beyer et al., 2009). Examples for non-thermal modifications of mycotoxins are the formation of hydrolysed fumonisins, degradation reactions induced by UV light for OTA and CIT (Schmidt-Heydt et al., 2012) or DON sulphonate generated by treatment of contaminated feed with sodium bisulfite (Danicke et al., 2010).

1.3 LEGISLATION

Since it is unrealistic to avoid the formation of mycotoxins, governmental regulatory agencies survey for the occurrence of mycotoxins in food and feed and establish regulatory limits to control the mycotoxin exposure of the population. The establishment of the maximum limits is based on epidemiological data and extrapolations from animal models, taking into account the different uncertainties associated with both types of analysis. Various factors affect the promulgation of mycotoxin limits and regulations such as the availability of toxicological and exposure data, the knowledge of mycotoxin distribution within commodities or the availability of analytical methods for mycotoxins (Van Egmond et al., 2007).

Current EU legislation includes maximum levels of aflatoxins, fumonisins, OTA, DON and ZEN in foods (1881/2006/EC), AFB_1 in feed (2002/32/EC) and guidance values for fumonisins, OTA, DON and ZEN in products intended for animal feeding (2006/576/EC). Recently, indicative

values regarding the occurrence of T-2 and HT-2 toxin (HT-2) in cereals and cereal products in food and feed (2013/165/EC) were established and in 2014 maximum levels of the contaminant citrinin in food supplements based on rice fermented with red yeast *Monascus purpureus* were included (212/2014/EC). Table 1.2. and table 1.3. give an overview of the maximum limits and guidance levels for mycotoxins in food and feed established by the European Commission. Regulations concerning sampling and analytical methods for the official control of mycotoxins in food and feed were established as well (401/2006/EC; 2002/657/EC). Current legislations are increasing based on scientific opinions of authoritative bodies such as the European Food Safety Authority (EFSA). At the same time, different European organisations and programs such as the Scientific Cooperation on Questions relating to Food (SCOOP), the Rapid Alert System for Food and Feed (RASFF), the creation of an EU Community Reference Laboratory for Mycotoxins (CRL) and the European Standardisation Committee (CEN) have a direct and indirect influence on the development of mycotoxin regulations. Until today no maximum limits or guidance values for mycotoxins in urine are available.

Table 1.2. Current maximum limits for mycotoxins in food according to 1881/2006/EC and indicative values for the sum of T-2 and HT-2 in food according to 2013/165/EC

	FOOD	µg/kg		
	Aflatoxins	**B_1**	**Sum of B_1, B_2, G_1 and G_2**	**M_1**
1	Groundnuts (peanuts) and other oilseeds, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs, with the exception of groundnuts (peanuts) and other oilseeds for crushing for refined vegetable oil production	8.0	15.0	—
2	Almonds, pistachios and apricot kernels to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	12.0	15.0	—
3	Hazelnuts and Brazil nuts, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8.0	15.0	—
4	Tree nuts, other than the tree nuts listed in 2 and 3, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	—
5	Groundnuts (peanuts) and other oilseeds and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs, with the exception of: crude vegetable oils destined for refining and refined vegetable oils	2.0	4.0	—
6	Almonds, pistachios and apricot kernels, intended for direct human consumption or use as an ingredient in foodstuffs	8.0	10.0	—
7	Hazelnuts and Brazil nuts, intended for direct human consumption or use as an ingredient in foodstuffs	5.0	10.0	—
8	Tree nuts, other than the tree nuts listed 6 and 7, and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2.0	4.0	—
9	Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	—
10	Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2.0	4.0	—
11	All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 12, 15 and 17	2.0	4.0	—
12	Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5.0	10.0	—
13	Raw milk, heat-treated milk and milk for the manufacture of milk-based products	—	—	0.050
14	Following species of spices: Capsicum spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika); Piper spp. (fruits thereof, including white and black pepper); Myristica fragrans (nutmeg); Zingiber officinale (ginger); Curcuma longa (turmeric) and mixtures of spices containing one or more of the abovementioned spices	5.0	10.0	—
15	Processed cereal-based foods and baby foods for infants and young children	0.10	—	—
16	Infant formulae and follow-on formulae, including infant milk and follow-on milk	—	—	0.025
17	Dietary foods for special medical purposes intended specifically for infants	0.10	—	0.025

	Citrinin	
1	Food supplements based on rice fermented with red yeast Monascus purpureus	2000
	Fumonisins	**Sum FB_1 and FB_2**
1	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	4 000
2	Maize intended for direct human consumption, maize-based foods for direct human consumption, with the exception of foodstuffs listed in 3 and 4	1 000
3	Maize-based breakfast cereals and maize-based snacks	800
4	Processed maize-based foods and baby foods for infants and young children	200
5	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	1 400
6	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	2 000
	Ochratoxin A	
1	Unprocessed cereals	5.0
2	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs listed in 9 and 10	3.0
3	Dried vine fruit (currants, raisins and sultanas)	10.0
4	Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5.0
5	Soluble coffee (instant coffee)	10.0
6	Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine	2.0
7	Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2.0
8	Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2.0
9	Processed cereal-based foods and baby foods for infants and young children	0.50
10	Dietary foods for special medical purposes intended specifically for infants	0.50
11	*Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika); *Piper* spp. (fruits thereof, including white and black pepper); *Myristica fragrans* (nutmeg); *Zingiber officinale* (ginger); *Curcuma longa* (turmeric); Mixtures of spices containing one or more of the abovementioned spices	15
12	Liquorice (Glycyrrhiza glabra, Glycyrrhiza inflate and other species) - Liquorice root, ingredient for herbal infusion - Liquorice extract for use in food in particular beverages and confectionary	20 80

	Deoxynivalenol	
1	Unprocessed cereals other than durum wheat, oats and maize	1 250
2	Unprocessed durum wheat and oats	1 750
3	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	1 750
4	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 7, 8 and 9	750
5	Pasta (dry)	750
6	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
7	Processed cereal-based foods and baby foods for infants and young children	200
8	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 19041010	750
9	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	1 250
	T-2 and HT-2 toxin	**Sum T-2 and HT-2**
1	Unprocessed cereals	
	- barley (including malting barley) and maize	200
	- oats (with husk)	1000
	- wheat, rye and other cereals	100
2	Cereal grains for direct human consumption	
	- Oats	200
	- Maize	100
	- Other cereals	50
3	Cereal products for human consumption	
	- oat bran and flaked oats	200
	- cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100
	- other cereal milling products	50
	- breakfast cereals including formed cereal flakes	75
	- bread (including small bakery wares), pastries, biscuits, cereal snacks, pasta	25
	- cereal-based foods for infants and young children	15
	Zearalenone	
1	Unprocessed cereals other than maize	100
2	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	350
3	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 6, 7, 8, 9 and 10	75
4	Refined maize oil	400

5	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50
6	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100
7	Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children	20
8	Processed maize-based foods for infants and young children	20
9	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	200
10	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	300

Table 1.3. Current maximum limits for AFB_1 in feed according to 2002/32/EC, guidance values for mycotoxins in feed according to 2006/576/EC and indicative values for the sum of T-2 and HT-2 in feed according to 2013/165/EC

	FEED (values relative to a feeding stuff with a moisture content of 12 %)	mg/kg
	Aflatoxins	**AFB_1**
1	Feed materials	0.02
2	Complete feedingstuffs for cattle, sheep and goat with the exception of:	0.02
	- Complete feedingstuffs for dairy animals	0.005
	- Complete feedingstuffs for calves and lambs	0.01
3	Complete feedingstuffs for pigs and poultry (except young animals)	0.02
4	Other complete feedingstuffs	0.01
5	Complementary feedingstuffs for cattle, sheep and goats (except complementary feedingstuffs for dairy animals, calves and lambs)	0.02
6	Complementary feedingstuffs for pigs and poultry (except young animals)	0.02
7	Other complementary feedingstuffs	0.005
	Fumonisins	**Sum FB_1 and FB_2**
1	Feed materials: Maize and maize products	60
2	Complementary and complete feedstuffs for:	
	- Pigs, horses (Equidae), rabbits and pet animals	5
	- Fish	10
	- Poultry, calves (< 4 months), lambs and kids	20
	- Adult ruminants (> 4 months) and mink	50
	Ochratoxin A	
1	Feed materials: Cereals and cereal products	0.25
2	Complementary and complete feedstuffs:	
	- Complementary and complete feeding stuffs for pigs	0.05
	- Complementary and complete feeding stuffs for poultry	0.1

	Deoxynivalenol	
1	Feed materials:	
	- Cereals and cereal products with the exception of maize by-products	8
	- Maize by-products	12
2	Complementary and complete feedstuffs with the exception of:	5
	- Complementary and complete feedstuffs for pigs	0.9
	- Complementary and complete feedstuffs for calves (< 4 months), lambs and kids	2
	T-2 and HT-2 toxin	**Sum T-2 and HT-2**
1	Cereal products for feed and compound feed:	
	- Oat milling products (husks)	2
	- Other cereal products	0.5
	- Compound feed, with the exception of feed for cats	0.25
2	Compound feed for cats	0.05
	Zearalenone	
1	Feed materials:	
	- Cereals and cereal products with the exception of maize by-products	2
	- Maize by-products	3
2	Complementary and complete feedstuffs:	
	- Complementary and complete feedstuffs for piglets and gilts (young sows)	0.1
	- Complementary and complete feedstuffs for sows and fattening pigs	0.25
	- Complementary and complete feedstuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5

CHAPTER 2

HEALTH EFFECTS OF MYCOTOXINS

Toxicokinetics studies the changes of mycotoxin concentrations in the body in function of time. These changes depend on the degree and the speed of four processes namely absorption, distribution, metabolism and excretion (ADME). The ADME processes are presented in figure 2.1.

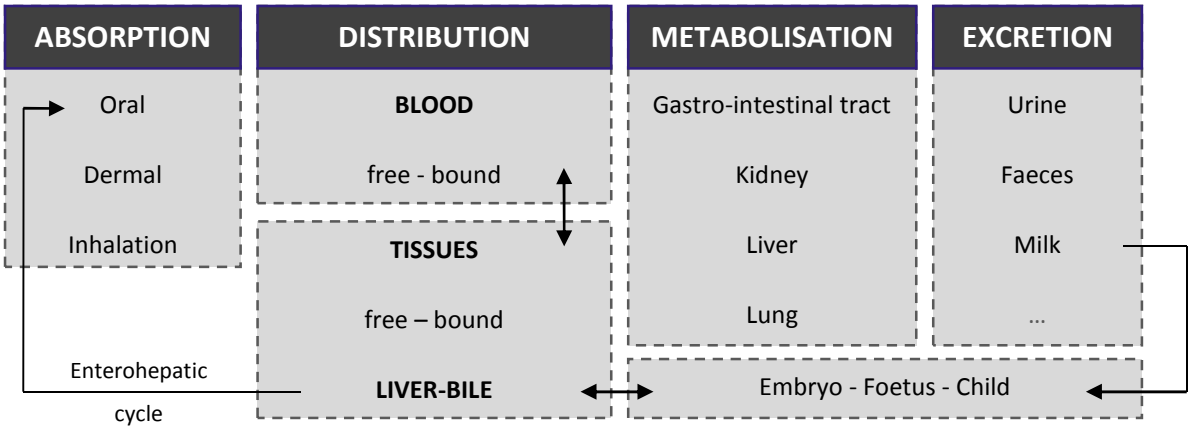


Figure 2.1. Toxicokinetic processes

Most of the toxicokinetic studies for mycotoxins are performed in animal studies. In contrast, the knowledge of these processes for mycotoxins in humans is until today not well developed. **Absorption** of mycotoxins occurs mainly through the gastro-intestinal tract via ingested contaminated food. The exposure of mycotoxins through residues in animal derived food is much lower in comparison with exposure through vegetable food. Absorption through inhalation or skin contact occurs to a lesser extent. Moreover, an enterohepatic cycle exists for mycotoxins, i.e. recycling via biliary excretion (Boonen et al., 2012; Pfeiffer et al., 2011; Ringot et al., 2006). After absorption mycotoxins are **distributed** over the body through the blood where mycotoxins can bind to red blood cells or to plasma proteins like

albumin. Studies on the distribution of mycotoxins to tissues is rather scarce. Most of the mycotoxins have a plasma half-life of 24 to 48 hours, except for OTA. **Elimination** of mycotoxins occurs through biotransformation and excretion. **Biotransformation** is the chemical transition to a more hydrophilic metabolite before excretion takes place. Enzymes of the liver play an important role in biotransformation. Biotransformation can lead to a bioactivation or detoxification. **Excretion** is the removal of the component out of the body. The main routes of mycotoxin excretion are via the kidneys (urine) or via the bile (faeces). Excretion through air, via salivary-, perspiratory- and mammary glands is negligible (Malekinejad et al., 2005). Toxicokinetic parameters differ within and between humans. For example children, adults and elderly show differences in absorption, metabolisation and excretion. Also the influence of several diseases or the intake of drugs on the kinetics of mycotoxins needs more research.

Toxicodynamics studies the interaction of a mycotoxin with biological targets and their downstream biological effects. For most of the mycotoxins the liver and the kidney are the target organs (Bennett and Klich, 2003; Fodor et al., 2008). Furthermore, mycotoxins can be carcinogenic, immunosuppressive, teratogenic or mutagenic.

This chapter gives an overview of the toxicokinetics, with main focus on biotransformation and excretion, and toxicological effects of each mycotoxin in humans. When no data are available in humans, results from studies performed in pigs are mentioned because of the given anatomical and physiological similarity of the gastro-intestinal tract, liver and kidneys.

2.1 AFLATOXINS

2.1.1 Toxicokinetics

AFB_1 absorption, distribution and elimination is rapid. AFB_1 is well absorbed and accumulates in liver where it is extensively metabolised. Aflatoxins are metabolised in the body by a variety of routes that can lead to either activation, i.e. the formation of aflatoxin 8,9-epoxide that binds to proteins and DNA or detoxification, being the formation of metabolites that are more easily excreted. There are four metabolic pathways of AFB_1: (1) O-dealkylation to aflatoxin P_1 (AFP_1), (2) ketoreduction to aflatoxicol (AFL), (3) epoxidation to AFB_1-8,9-epoxide and (4) hydroxylation to AFM_1, aflatoxin Q_1 (AFQ_1) and aflatoxin B_{2a} (AFB_{2a}). Cytochrome P450 enzymes (CYP), more precisely CYP3A4 and 1A2 in humans, convert aflatoxins to the reactive 8,9-epoxide form (Eaton and Gallagher, 1994; Gallagher et al., 1994). CYP1A2 metabolises AFB_1 to AFB_1-endo-8,9-epoxide which is less toxic considering that it cannot bind nucleic acids and can be excreted under different forms. Both the exo- and endo-epoxides can undergo rapid non-enzymatic hydrolysis to AFB_1-8,9-dihydrodiol, reacting with the aminogroup of lysine in serum albumin. Detoxification of the epoxide can also occur enzymatically via conjugation to glutathione, mediated by glutathione-S-transferase found in the cytosol and microsomes, leading to the excretion of aflatoxin (Calderone and Cihlar, 2002; Raj et al., 1986). Figure 2.2. gives an overview of the different biotransformation pathways of AFB_1. The parent compound is excreted in faeces and unbound water-soluble metabolites are excreted in urine (Riley et al., 2011).

Literature data has shown that AFB_1 can be transferred through human placenta and metabolised by local enzymes. The placenta contains low levels of CYP enzymes, with variations depending on the stage of placental development. Furthermore, AFM_1 is a frequent metabolite in milk of exposed lactating animals, including in human breast milk after maternal exposure to dietary AFB_1 (Wild and Gong, 2010).

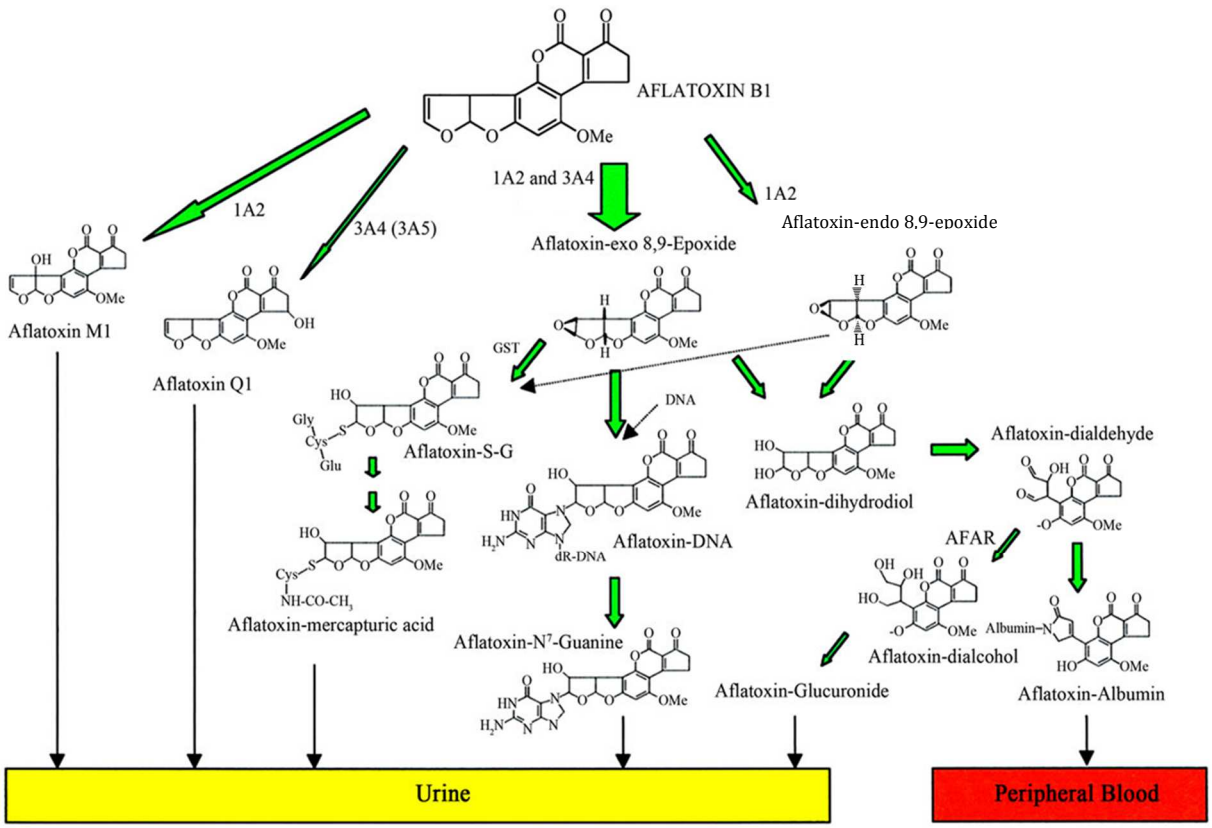


Figure 2.2. Metabolic pathway of AFB_1 in animals and human leading to reactive metabolites and biomarkers. (1A2: CYP1A2; 3A4: CYP3A4; 3A5: CYP3A5; GST: glutathione S-transferase; AFAR: aflatoxin aldehyde reductase; Aflatoxin-S-G: aflatoxin-glutathione conjugate)(Wild and Turner, 2002).

2.1.2 Toxicity

The diseases caused by aflatoxin consumption are called aflatoxicoses. Acute aflatoxicosis results in death whereas chronic aflatoxicosis results in cancer and immune suppression (Hsieh, 1988). The liver is the primary target organ and the magnitude of the response is influenced by several factors such as age, sex or the presence of other mycotoxins and pharmacologically active substances (Newberne and Butler, 1969). Aflatoxins are classified as group 1 carcinogenic for humans by the International Agency of Research on Cancer (IARC). AFB_1 and AFG_1 possess an unsaturated bond at the 8,9 position on the terminal furan ring and subsequent studies demonstrated that epoxidation at this position and the formation of mutagenic DNA adducts is the key initiating step in the carcinogenesis of aflatoxins (Groopman and Kensler, 2005; Wild and Turner, 2002). AFB_2 and AFG_2 are

relatively non-toxic because of the lack of the unsaturated bond at the 8,9 position unless they are first metabolically oxidized to AFB_1 and AFG_1 *in vivo*. Mechanistically, it is known that the reactive aflatoxin epoxide binds to the N^7 position of guanines in DNA. Moreover, AFB_1-DNA adducts can result in guanine-cytosine to thymine-adenine transversions (Cullen and Newberne, 1994).

Because of the differences in aflatoxin susceptibility in laboratory animals, it has been difficult to extrapolate the possible effects of aflatoxin to humans. It has been hypothesised that kwashiorkor, a severe malnutrition disease, may be a form of pediatric aflatoxicosis (Hendrickse, 1997). Further early speculations that aflatoxin might be involved in Reye's syndrome, an encephalopathy, and fatty degeneration of the viscera in children and adolescents have not been substantiated (Hayes, 1980). Furthermore, aflatoxins cause growth suppression in animals and limited evidence suggests that such effects may also occur in humans. Gong et al. (2004) performed a study in Benin whereby a strong negative correlation was found between aflatoxin-albumin adducts and height of children. Turner et al. (2007) demonstrated a strong effect of maternal aflatoxin exposure during pregnancy on the growth in the first year of life in Gambia. The underlying mechanisms of the effect of aflatoxin exposure during infancy on faltering growth remains unclear. Exposure to aflatoxins in the diet is considered as an important risk factor for the development of primary hepatocellular carcinoma, particularly in individuals already exposed to hepatitis B. In different animal studies aflatoxin induced a high incidence of hepatocellular carcinoma, suspecting that this agent could contribute to human cancer (Eaton and Gallagher, 1994; Gallagher et al., 1994). In classical epidemiology, several studies have linked liver cancer incidence to estimated aflatoxin consumption in the diet. For example, Peers and Linsell (1973) and Van Rensburg et al. (1985) performed studies in Kenya and Mozambique whereby a positive correlation was found between dietary aflatoxin intake and hepatocellular carcinoma. The results of these studies have not been entirely consistent, and quantification of lifetime individual exposure to aflatoxin is extremely difficult.

2.2 CITRININ

2.2.1 Toxicokinetics

Specific toxicokinetic studies with oral administration are not available for CIT. CIT is eliminated predominantly by renal excretion, as described in a study with radiolabelled CIT by Reddy et al. (1982) in which approximately 75 % of the intraperitoneal dose was recovered in urine of rats. Approximately 74 % of the radioactivity appeared in the urine in the first 24 h, with only 1.7 % and 1.4 % in the urine at 48 h and 72 h, respectively. Sandor et al. (1991) described a subacute toxicity study with OTA and CIT in pigs. In this limited study, groups of three animals were given CIT, OTA or both compounds and compared with a group of five control animals. The authors concluded that CIT is more rapidly eliminated than OTA. No toxicokinetic studies have been conducted in humans.

2.2.2 Toxicity

Studies on the mechanism of CIT toxicity were performed exclusively *in vitro*. Inhibition of ribonucleic acid (RNA) and DNA synthesis by CIT has been reported in different mammalian cell lines including kidney cells (Wasternack and Weisser, 1992; Yoneyama and Sharma, 1987). Only few studies on adverse effects of CIT in pigs could be identified. At present, no effect has been reported from pigs given 20 µg/kg body weight (BW) per day (Sandor et al., 1991). EFSA considered this intake value as a no observed adverse effect level (NOAEL), which is consistent with the results from a subchronic study in rodents (Lee et al., 2010). Available animal studies revealed that CIT induces renal adenomas and chromosome abnormalities. However as no lifetime exposure studies are available, no conclusion can be drawn regarding the potential carcinogenicity of CIT. In repeated dose toxicity studies, the kidney was identified as the principal target organ for CIT and significant species differences in the susceptibility to CIT have been observed. Studies of the immunotoxicity of CIT are rather incomplete, often non-specific and do not allow a conclusive evaluation. *In vitro* and

in vivo studies provided clear evidence for reproductive toxicity and teratogenic and embryotoxic effects of CIT. Furthermore, CIT has multiple effects on mitochondrial function, impairing mitochondrial respiration with subsequent loss in cellular adenosine triphosphate (ATP) levels. Whether alteration of mitochondrial function is a primary or contributing mechanism to cell death by CIT remains to be clarified (Segvic et al., 2012).

2.3 FUMONISINS

2.3.1 Toxicokinetics

Toxicokinetic studies indicated that FB_1 is poorly absorbed by the gastro-intestinal tract and rapidly cleared from the plasma in laboratory animals. FB_1 doesn't extensively accumulate in tissues although low levels of FB_1 were found in the liver and kidney (Norred et al., 1996; Shephard et al., 1995). Following intragastric dosing, FB_1 appeared in the blood after 30 min and was mainly eliminated in the faeces within 72 hours. Less than 1 % was excreted in urine and an enterohepatic recirculation contributes to a longer half-life of the mycotoxin. Only a few data have been reported in literature about the metabolism of FB_1 (Marasas et al., 2001). An *in vivo* experiment performed by Fodor et al. (2008) in pigs showed the transformation of FB_1 by intestinal microbiota to partially hydrolysed FB_1 and hydrolysed FB_1 (HFB_1 or aminopentol). HFB_1 was less potent than FB_1 as an inhibitor of ceramide synthase *in vitro* but its derivative appeared to be tenfold more toxic than FB_1 .

2.3.2 Toxicity

The chemical structure of FB_1 is similar to sphingosine (So) and sphinganine (Sa), the backbones of sphingolipids (SL). As result of this similarity, FB_1 inhibits the enzyme ceramide synthase, a key enzyme in the SL metabolism. The primary amine function appears necessary for its biological activity. Inhibition of ceramide synthase results in a decrease of *de novo* synthesis of ceramide and SL (Dragan et al., 2001). The blocking of this pathway by FB_1 leads

to an increase in free sphinganine and to a lesser extent in sphingosine (figure 2.3.). SL's are important constituents of cell membranes and by impairing SL metabolism FB_1 alters the membrane functions. One affected function is the folate receptor function which possibly causes the inhibition of folate intake and possible neural tube defects (NTD). Different animal studies showed that the brain SL metabolism was disturbed after FB_1 dosing, which might suggest that the disturbed SL metabolism is responsible for the FB_1 toxicity (Kwon et al., 1997). Furthermore, FB_1 blocks the mitochondrial respiration by inhibiting the mitochondrial electron transport chain. This leads to depolarisation of the membrane, reactive oxidative species (ROS) over-production and calcium deregulation, resulting in cell death (Domijan, 2012).

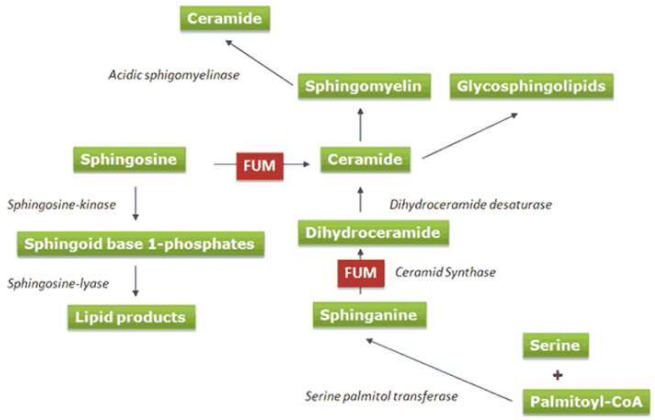


Figure 2.3. Sphingolipid metabolism showing the inhibition of ceramide synthase by fumonisins and the changed concentrations of other compounds caused by this inhibition (Merrill et al., 2001)

FB_1 is a neurodegenerative mycotoxin and ingestion of FB_1 contaminated feed has been associated with pulmonary oedema in pigs (Harrison et al., 1990) and equine leukoencephalomalacia (ELEM), a brain disorder in horses (Marasas, 1996). In animal models, FB_1 was shown to be neurotoxic, carcinogenic and immunotoxic. The most affected organs are the kidneys and the liver (Gelderblom et al., 1991). In certain regions of the world, where maize is a staple food, higher incidence of oesophageal cancer was connected with dietary exposure to FB_1. For this reason, IARC classified FB_1 in group 2B as a possible carcinogen to

humans. However, epidemiological data are still lacking and a direct causal role has not been established.

2.4 OCHRATOXINS

2.4.1 Toxicokinetics

OTA is passively absorbed in the gastro-intestinal tract and has a high affinity for plasma proteins. Also reabsorption of OTA from the intestine, enterohepatic recirculation and reabsorption in the kidney proximal and distal tubulus favor its accumulation in the body (Dahlmann et al., 1998). In humans, OTA has a serum half-life of about 35 days (Schlatter et al., 1996). Different studies in human showed that the OTA concentration in foetal serum was higher than the maternal one, indicating an active placental transfer (Miraglia et al., 1998). Furthermore, several studies reported OTA levels in breast milk suggesting OTA transfer to milk (Munoz et al., 2013). Biotransformation of OTA has not been elucidated in detail. The major metabolic pathway is the hydrolysis into a less toxic compound, ochratoxin α (OTα) mainly through the intestinal microflora. In addition, a small percentage of absorbed OTA is hydroxylated into 4-hydroxy-OTA (4-OH-OTA) through phase I detoxification reactions whereby two epimers are formed (Marquardt and Frohlich, 1992). The 4(R)-OH-OTA epimer is mainly formed by human CYP3A4, CYP1A2 and CYP2C9 and is less toxic than OTA. In some animal species also 10-hydroxy-OTA (10-OH-OTA) production has been described. OTC is a naturally occurring toxin and can be converted to OTA in the body. Additionally OTA can be conjugated with glucuronic acid and sulphates during phase II metabolisation. The absence of these conjugates in several studies remains an open question (Gross-Steinmeyer et al., 2002). In all species faecal and urinary excretions play important roles in plasma clearance of OTA. Renal excretion occurs through tubular elimination and is carried out by several transporter proteins. Both OTA and its metabolite OTα are excreted in the faeces due to the biliary excretion (Ringot et al., 2006).

2.4.2 Toxicity

Various studies in animals and humans have associated OTA with endemic porcine nephropathy and Balkan endemic nephropathy (Pfohl-Leszkowicz and Manderville, 2007). Kidneys are the main target organ for OTA and its immunosuppressive properties result in higher susceptibility to infections. Furthermore, OTA is teratogenic and IARC classified OTA in group 2b as a possible carcinogen for human. OTA inhibits protein synthesis by competing with phenylalanine (Creppy et al., 1995). Conflicting data exists on the genotoxicity of OTA and in particular its mode of action. Increasing evidence suggests that the various genetics effects seen in *in vitro* and *in vivo* studies are compatible with the hypothesis of DNA damage induced by oxidative stress rather than indicating direct interaction (adduct formation) of OTA with cellular DNA. Furthermore, chemical analysis failed to identify specific DNA adducts of OTA or its metabolites (EFSA, 2006).

2.5 TRICHOTHECENES

2.5.1 Toxicokinetics

In general, the available information on toxicokinetics of T-2 is incomplete for each animal species. Up to now, five biotransformation pathways of T-2 toxin, involving hydrolysis, hydroxylation, de-epoxidation, glucuronidation and acetylations, have been described in different biological systems, resulting in a large number of different metabolites. The main metabolic pathway in all species is via a rapid deacetylation in C-4, resulting in the formation of HT-2. This reaction is catalysed by a non-specific carboxyesterase found in blood plasma and in several tissues, primarily in the liver. Depending on the metabolic pathway, HT-2 can be further deacetylated, hydroxylated or conjugated. The metabolism of T-2 in human skin revealed three major metabolites namely HT-2, T-2 triol and T-2 tetraol. The minor metabolic pathway (hydroxylation of T-2) is an activation reaction since 3-hydroxy-T-2 was found to be more toxic (Wu et al., 2010).

The toxicokinetics of DON have been established in rats and pigs, but are not yet fully understood for humans. DON is detectable in serum in high amounts immediately after ingestion, but is rapidly cleared from the blood stream. The main fraction is excreted by urine and a smaller part is eliminated in faeces (Turner, 2010). DON has a short half-life because of its fast excretion (4 to 8h). Generally, DON is rapidly metabolised and does not accumulate in tissues (Pestka, 2010). Recently, the first *in vivo* case study providing detailed information about the human metabolism of DON in one volunteer was published (Warth et al., 2013a). DON, DON3GlcA and DON15GlcA were detected in human urine whereby 68 % of the ingested dose was excreted in urine. None of these toxins were detectable after 24 hours after exposure, which suggests a rapid metabolism of DON. The excretion of DON and its glucuronides occurred within 3-5 hours after consumption of lunch. The glucuronidation ratio was calculated to be 76 % with DON15GlcA as main DON metabolite found in human urine samples (73 %). Besides DON3GlcA and DON15GlcA, a third DON metabolite was found with large similarities to the spectra of the other DON glucuronides, suggesting the presence of deoxynivalenol-7-glucuronide (Maul et al., 2012).

2.5.2 Toxicity

Toxicodynamic studies have demonstrated that the trichothecenes function as inhibitor of eukaryotic protein synthesis. Trichothecenes bind to the 60S-ribosomal subunit and interact with peptidyltransferase (figure 2.4.). This interaction leads to the inhibition of the polypeptide chain initiation (T-2) or elongation-termination (DON) (Pestka and Smolinski, 2005). The toxic properties of trichothecenes reside in the common 12,13 epoxide group that allows them to inhibit protein synthesis. T-2 is the strongest protein synthesis inhibitor, whereas DON is less potent. Addition of an acetyl chain (see 2.7.) can further influence its inhibitory potential (Ueno, 1983).

The toxic effects are known to involve ribotoxic stress responses. Trichothecenes activate mitogen-activated protein kinases (MAPKs) of a signaling cascade that regulates cell survival

in response to stress and induces rapid apoptosis (Moon and Pestka, 2003; Pestka et al., 2004).

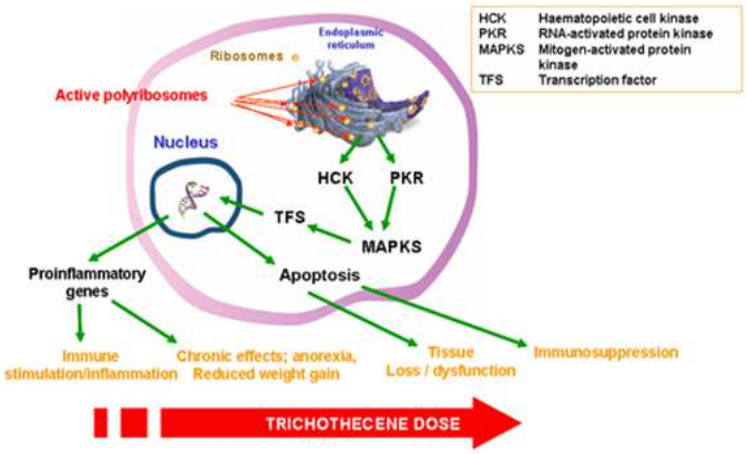


Figure 2.4. Mechanism involved in trichothecene induced toxicity (Pestka, 2007). Trichothecenes function as inhibitor of eukaryotic protein synthesis as they bind to the 60S-ribosomal subunit and interact with peptidyltransferase. Trichothecenes activate mitogen-activated protein kinases of a signaling cascade that regulates cell survival in response to stress and induces rapid apoptosis.

The acute toxicity of trichothecenes results in nausea, vomiting, diarrhea and gastro-enteric distress. Cells of the immune system are central targets of the trichothecenes. Pathophysiologically, DON has been linked to anorexia, growth retardation and immunotoxicity in animal models. It is yet to be established whether DON is related to certain chronic conditions in humans, such as colitis or inflammatory bowel syndrome. However, so far it has been shown that DON has no carcinogenic effects, neither in animals nor humans. IARC has listed trichothecenes as group 3 non-carcinogenic. T-2 is the most acutely toxic among the trichothecenes. For most of the metabolites of T-2 toxin, no or very limited toxicological information is available. Nevertheless, the de-epoxidation is considered to be an important detoxification step. Furthermore, T-2 and HT-2 are able to penetrate across the blood brain-barrier *in vivo* (Weidner et al., 2013).

2.6 ZEARALENONE

2.6.1 Toxicokinetics

Recently, the first *in vivo* case study providing detailed information about the human metabolism of ZEN in one volunteer was published (Warth et al., 2013a). 9.4 % of ZEN was excreted in the urine after the ingestion of naturally contaminated food. Furthermore, zearalenone-14-glucuronide (ZEN14GlcA) was detected in urine samples 3-10 hours after lunch, which indicates a rapid formation and excretion of ZEN14GlcA. ZEN is metabolised by 3α- and 3β-hydroxysteroid dehydrogenase into α- and β-zearalenol (α- and β-ZEL), respectively. β-ZEL has lower affinity for the estrogen receptor, whereas α-ZEL has higher binding affinity compared to ZEN. The metabolisation to β-ZEL can be seen as a detoxification step, whereas the metabolisation to α-ZEL leads to bioactivation (Malekinejad et al., 2005). Also phase II metabolisation reactions take place whereby ZEN and its metabolites are conjugated with glucuronic acid, catalysed by uridine diphosphate glucuronyl transferases. Glucuronidation enhances the water solubility of compounds, thus enhancing renal elimination (Pfeiffer et al., 2010). On the other hand, enterohepatic recycling occurs by biliary excretion followed by an intestinal deconjugation and reabsorption, which has been demonstrated for ZEN (Biehl et al., 1993).

2.6.2 Toxicity

ZEN exerts its toxic action by passively crossing the cell membrane and competitively binding to the estrogen-receptor. The formed ligand-receptor-complex is transferred to the nucleus where it binds to specific nuclear receptors, and generates estrogenic responses via gene activation (figure 2.5.) (Metzler et al., 2010; Riley and Norred, 1996).

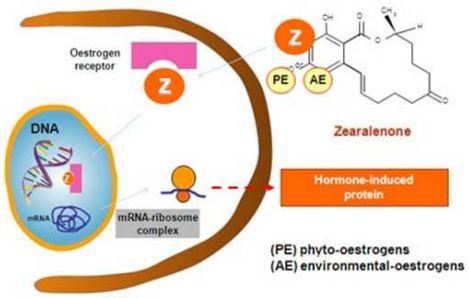


Figure 2.5. Mechanism involved in ZEN induced toxicity in animals (Riley and Norred, 1996). ZEN exerts its toxic action by passively crossing the cell membrane and competitively binding to the estrogen-receptor. The formed ligand-receptor-complex is transferred to the nucleus where it binds to specific nuclear receptors, and generates estrogenic responses via gene activation.

ZEN is involved in reproductive disorders in animals and hyperestrogenic conditions in humans. The estrogenic effects on humans and animals are a result of interaction of the toxin or its metabolites with the estrogen receptor. They are structurally similar to 17β-estradiol and therefore act as estrogenic agonists. Human studies have shown that ZEN may be involved in the etiology of breast cancer (Yu et al., 2005) and may affect humans in the pre-pubertal stage (Massart and Saggese, 2010). Besides its endocrine disrupting and anabolic effects, ZEN was reported to induce hepatocellular lesions in animal models, which are likely to lead to the development of local adenomas. The central nervous system consists of other α- and β-estrogen receptors and effects on this level are reported as ZEN is able to pass the blood-brain barrier, still the ovaria and uterus are more sensitive. IARC has listed ZEN as group 3 non-carcinogenic.

2.7 MODIFIED MYCOTOXINS

Potential exposure to modified mycotoxins due to their presence in food and feed raises concern that modified mycotoxins may pose an additional risk to human and animal health. Conjugated and matrix-associated mycotoxins may be cleaved by the gut microflora to the parent compound and thus add to the systemic exposure and toxicity of the free mycotoxin.

Therefore it is critical to assess the bioavailability and toxic potential of modified mycotoxins (Rychlik et al., 2014).

Only a few *in vivo* data on ADME processes and toxicity of modified mycotoxins are yet available. The best studied examples so far involve derivatives of DON and ZEN namely DON3Glc, 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and ZEN14Glc. *In vitro* studies on the comparative toxicity of DON and its acetylated derivatives demonstrate that 15ADON is more toxic and 3ADON is less toxic compared to DON. DON3Glc may be hydrolysed to DON in the gastro-intestinal tract and thus may contribute to the total dietary exposure to DON (Nagl et al., 2014). Therefore, it may be concluded that data on the toxic potential of DON3Glc using cellular or molecular endpoints *in vitro* such as inhibition of protein synthesis may not be accurate predictors of systemic toxicity of DON3Glc in animals or humans. DON is nearly completely modified to DOM-1 in cattle by rumen micro-organisms prior to systemic absorption. This modification occurs also in pigs, but to a lesser extent as most of the DON is already absorbed before reaching the intestinal segment where DON is converted to DOM-1. Therefore, in DON-exposed cattle, mostly DOM-1 is detectable in blood while in pigs the majority is in the form of free DON (Daenicke and Brezina, 2013). Similarly to DON3Glc, glycosylation of the estrogenic mycotoxin ZEN by plants to its derivative ZEN14Glc has been shown to prevent binding to estrogen receptors (Poppenberger et al., 2006). It is important for human and animal health risk assessment to consider that ZEN14Glc may be cleaved during digestion and release its active parent compound as demonstrated in pigs (Gareis et al., 1990). These examples emphasize the need for toxicokinetic and toxicity data on modified mycotoxins to allow human and animal health risk assessment (Rychlik et al., 2014).

CHAPTER 3

MYCOTOXIN EXPOSURE ASSESSMENT: FRAMEWORK AND METHODOLOGICAL ASPECTS

3.1 THE GENERAL FRAMEWORK OF RISK ASSESSMENT

Food contains a wide range of substances which are either desired (nutrients, additives) or undesired, such as mycotoxins. As a result, both nutritional and food safety issues are important determinants of public health and therefore deserve continued attention from scientific and from policy perspective. A very important aspect within mycotoxin research is risk assessment. According to the International Programme on Chemical Safety (IPCS, 2004) health risk is defined as the probability of an adverse effect in an organism, system or population caused under specified circumstances by exposure to an agent. The use of a structured risk analysis process facilitates decision-making in the area of food safety. Risk analysis has been defined by the Codex Alimentarius Commission as a process consisting of three components: risk assessment, risk management and risk communication. Within risk analysis, the functional separation between risk assessors and risk managers is essential to ensure scientific objectivity of the risk assessment process (FAO/WHO, 2008).

Risk assessment of food chemicals can be generally described as characterising the potential hazards and the associated risks to life and health resulting from exposure of humans to chemicals present in food over a specified period. It provides a mechanism for the structured review of information relevant to estimating health outcomes in relation to exposure to chemicals present in food and forms the scientific basis for the risk management executed by governments. Risk assessment is defined as a four-step process (figure 3.1.). Within this chapter the different steps will be explained with a direct link to the scope of this doctoral thesis.

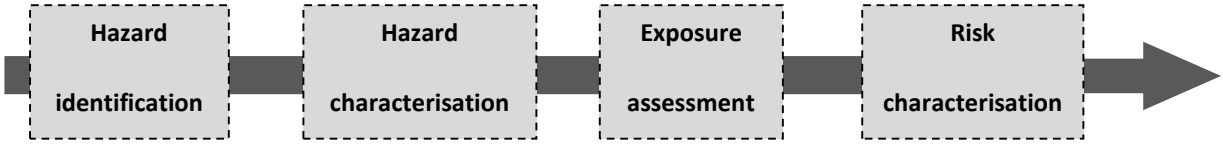


Figure 3.1. Four-step process of risk assessment

Hazard identification is the first of four steps in risk assessment. The purpose of food chemical hazard identification is to evaluate the weight of evidence for adverse health effects, based on assessment of all available data on toxicity and mode of action. It is designed to address two questions namely the nature of the health hazard that an agent may pose and the circumstances under which the hazard may be expressed. The nature of the toxicity or adverse health effect and the affected target organs are identified using *in vitro* and *in vivo* toxicity tests. The hazard identification concerns the determination of the different compounds to be included in the analysis of the risks related to mycotoxins. Although a very wide range of mycotoxins may accumulate in the food chain, it was decided to limit this study to the most abundant mycotoxins with emphasis on those with available human biomarkers of exposure. For this reason aflatoxins, CIT, fumonisins, ochratoxins, trichothecenes and ZEN were included in this study whereby dietary intake is the main route of exposure (see chapter 1).

Hazard characterisation describes the relationship between the dose/exposure and the incidence of the adverse health effect (dose-response relationships) and includes all toxicokinetic and toxicodynamic data concerning mycotoxins (see chapter 2). In cases where the toxic effect is assumed to have a threshold, hazard characterisation usually results in the establishment of health-based guidance values such as a tolerable weekly intake (TWI) or a tolerable daily intake (TDI). This establishment is based on the determination of a NOAEL in toxicological studies and the application of an uncertainty factor. The uncertainty factor means that the lowest NOAEL in animal studies is divided by 100, 10 for extrapolation from animals to humans and 10 for variation between individuals, to arrive at a tolerable intake

level. In cases where the data are inadequate, higher safety factors are used (FAO, 2003). Table 3.1. gives an overview of the health-based guidance levels for mycotoxins in humans.

Table 3.1. Health-based guidance levels for mycotoxins in humans

Mycotoxin	Health-based guidance level	Reference
Aflatoxins	No TDI since carcinogenic effects	
Fumonisins	Group TDI = 2 µg/kg BW/day	FAO/WHO (2012)
Ochratoxin A	TWI = 0.12 µg/kg BW/week	EFSA (2006)
Deoxynivalenol + acetylated forms	Group TDI = 1 µg/kg BW/day	FAO/WHO (2010)
T-2 + HT-2 toxin	Group TDI = 0.1 µg/kg BW/day	EFSA (2011a)
Zearalenone	TDI = 0.25 µg/kg BW/day	EFSA (2011b)

The largest part of the work executed in this PhD-thesis is related to the **exposure assessment**. The latter has been defined as the qualitative and/or quantitative evaluation of the likely intake of chemical agents via food as well as exposure from other sources if relevant (FAO/WHO 2008). In the case of food chemicals, dietary intake is usually estimated by models combining data on food consumption with concentration data measured in foods and food groups and the likelihood of consumers eating large amounts of the foods in question (high consumers) and of the chemical being present in these foods at high levels. Usually a range of intake or exposure estimates will be provided and estimates may be broken down by subgroup of the population (e.g. children, adults). Because of some uncertainties related to this approach, the direct measurement of biomarkers of exposure has become an added value in evaluating exposure. It is the only available tool that integrates exposures from all sources (Choi et al., 2015). Furthermore, the individual variation in ADME processes is integrated when using biomarkers, whereby a more accurate assessment of exposure can be performed at the individual level. The experimental part of this PhD-thesis focuses on the exposure assessment of mycotoxins for the Belgian population using biomarker analysis (see chapter 5-7).

The latter step, **risk characterisation**, integrates the information collected in the preceding three steps. The information from the intake or exposure assessment and the hazard characterisation is integrated into advice suitable for decision-making in risk management. Risk characterisation provides estimates of the potential risk to human health under different scenarios. It can be qualitative or quantitative. It should include a clear explanation of any uncertainties resulting from gaps in the science base and information on susceptible subpopulations. Finally the risk of mycotoxin exposure of the Belgian population was evaluated within this study. Based on the urinary mycotoxin concentrations, the dietary intake was estimated and was compared to TDI's (see chapter 6). The results of this PhD-thesis can help policy makers to get a better picture of the mycotoxin exposure of the Belgian population and to develop a strategy to tackle the potential public health problem related to mycotoxin exposure.

Risk assessment is followed by **risk management,** which is the process, distinct from risk assessment, of weighing policy alternatives, in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of consumers and if needed selecting appropriate prevention and control options. Risk managers are responsible for the final decisions on establishing maximum limits for contaminants such as mycotoxins.

Additionally **risk communication** is defined as the interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions (FAO/WHO 2008).

3.2 BIOMARKERS OF EXPOSURE

Biomarkers are measurable biochemical or molecular indicators of either exposure (biomarkers of exposure) or biological response (biomarkers of effect) to a mycotoxin that can be specifically linked to the proximate cause (Baldwin et al., 2011). Mechanism-based biomarkers often include changes in the level of specific proteins, cellular metabolites, or gene expression resulting from specific alterations in metabolic or signaling pathways, stress responses, cell proliferation, or cell death. Typical biomarkers of exposure are the parent toxins themselves, protein or DNA adducts and major phase I and II metabolites (e.g. glucuronide conjugates). These compounds can be detected in easily-accessed biological fluids such as urine and are related to the actual intake of the toxin through contaminated food. Ideally, such a marker should reflect the toxicokinetics, transformation and fate of the assessed contaminant in the body. Regardless of the type, all biomarkers should be specific, sensitive and as less invasive as possible (Crews et al., 2001). Sensitivity is related to the possibility of detection of the assessed contaminant or its metabolites when found in low amounts either in food or biological samples; the limit of detection (LOD) is an issue, considering that significant biological effects that may occur in humans following long term exposure to such low levels. Specificity is the certainty of associating the marker only to the exposed compound and not to other unrelated compounds. The most common parameters in quantifying exposure or effect are available from urine, serum and milk, although for some of the toxins there are a few other biological matrices that can provide us with important information, like faeces, nails or hair. Within this PhD thesis only biomarkers of exposure will be described with emphasis on urinary biomarkers.

3.2.1 Biomarkers for exposure to aflatoxins

By the late 1980's, two major biomarkers of aflatoxin exposure were developed: the urinary DNA adduct AFB_1-N^7-Guanine and the serum albumin adduct. The urinary DNA adduct reflects recent exposure as excretion occurs over 24-48 hours, whereas the albumin adduct reflects long-term exposure (2-3 months). Wild et al. (1986) showed that there was a

correlation between DNA adducts and serum albumin adducts in rats fed with AFB_1. Both biomarkers were further validated through population studies whereby a dose-dependent relationship between aflatoxin exposure and excretion in human urine and blood was shown (Egner et al., 2006; Groopman et al., 1993; Wild et al., 1992). Furthermore, correlations between ingestion of AFB1 and excretion of AFM1 in urine were observed in different populations (Zhu et al., 1987).

3.2.2 Biomarkers for exposure to citrinin

Data on biomonitoring of CIT are scarce because of the lack of suitable methods for human specimens. Recently, a few studies have been conducted whereby CIT and its metabolite dihydrocitrinone (HO-CIT) were analysed in human blood and urine (Blaszkewicz et al., 2013; Degen et al., 2014). Further research is needed to investigate if HO-CIT is a possible biomarker for CIT exposure in human and animals.

3.2.3 Biomarkers for exposure to fumonisins

FB_1 is detectable in urine, serum and faeces and it has been proposed as a biomarker of exposure, despite the fact that the detectable concentrations in all physiological fluids are quite low, approaching the LOD of the method used (Shephard et al., 2007). Moreover, the detection window of the toxin is very narrow, so only recent exposure can be quantified. Nevertheless, the correlation of this marker to fumonisin exposure needs to be further investigated in humans.

The disruption of sphingolipid metabolism results in an increase of sphinganine (Sa) levels and of sphinganine to sphingosine (So) ratio. Although older studies promote usage of Sa as biomarker of exposure to fumonisins, recently it has been suggested that Sa is not reliable in relation to the fumonisin exposure levels. The Sa:So ratio is a useful marker in animals, but it failed to correlate human exposure to fumonisins in a satisfactory way, probably because

the exposure in human population is generally low. Moreover, the normal variation of Sa:So ratio is considerably large and fluctuates over time regardless of the levels of exposure to the mycotoxin. Several studies conducted by the same author in Europe, Africa and South-America also show that the Sa:So ratio in urine and plasma may not be a relevant marker. Despite this, considering that most of the studies in literature promote these two parameters as valid in evaluating fumonisins effect, they cannot yet be excluded as biomarkers (Shephard et al., 2007; Van der Westhuizen et al., 2010).

Sewram et al. (2003) showed for the first time that FB_1, FB_2 and FB_3 can accumulate in human hair as a result of contaminated maize consumption. Hair analysis is relevant in measuring exposure to fumonisins regardless of source or intake profile, in terms of low, medium or high exposure. However, there is a big inter-individual variability, due to ethnicity, age, gender and other factors.

3.2.4 Biomarkers for exposure to ochratoxins

OTA is ubiquitous in human blood (serum and plasma), being an indicator of continuous exposure to this mycotoxin (Munoz et al., 2010). Despite the fact that urinary levels of OTA are considerably lower than in blood, following a study in the UK, urine was found to be a better marker of OTA intake (Gilbert et al., 2001). Around 50 % of the ingested OTA is excreted in urine, the rest being metabolites or conjugates of the toxin. The major metabolic pathway of OTA is represented by its hydrolysis to OTα. It is the only metabolite found so far in humans (Munoz et al., 2010).

OTA has been detected in various concentrations in breast milk of mothers in Europe. Nevertheless, up to the present time there is no correlation between OTA levels in breast milk compared to serum, suggesting that the transfer from blood to milk is not yet fully understood (Munoz et al., 2014; Munoz et al., 2013). Data regarding the ability of OTA or its metabolites to form adducts with nucleic acids is controversial. Certain studies have shown

formation of DNA adducts in humans (Pfohl-Leszkowicz et al., 2007). Until recently, studies suggested that OTA is poorly metabolised and does not form reactive intermediates capable of interacting with DNA (Mally et al., 2004).

3.2.5 Biomarkers for exposure to trichothecenes

Turner et al. (2008c) established that there is a quantitative correlation between urinary DON and the exposure to this mycotoxin. In human studies, the same authors identified urinary DON as being a reliable biomarker of exposure in UK and French populations (Turner et al., 2010a; Turner et al., 2010b). Moreover, the levels of DON in urine significantly correlated to the amount of contaminated cereals ingested one day prior to the urine collection.

So far, it is certain that there are two major metabolites of DON in mammals: DON glucuronide (DON3GlcA and DON15GlcA) and DOM-1 (Gratz et al., 2013; Warth et al., 2013a). DOM-1 is most likely generated by the intestinal microbiota of mammals and in particular in cattle where more than 95 % of DON is metabolised to DOM-1. Moreover, deepoxy-deoxynivalenol-glucuronide (DOMGlcA) is also ubiquitous in cows (Goyarts and Danicke, 2006; Pestka and Smolinski, 2005), being found in higher amounts than the parent compound. It is yet to be proven that DOM-1 is a relevant biomarker of exposure for DON in humans. Literature data suggests that DOM-1 is not a major metabolite in humans, considering that it is not found in all individuals (Gratz et al., 2013). The major metabolite of DON found in urine, both for animals and humans, are DON-glucuronides. DON-glucuronides have been previously found in urine and serum of pigs and recent data showed that in humans, about 91 % of the total urinary DON is represented by its glucuronide metabolites (Turner et al., 2010b). For T-2 and HT-2 no correlations between intake and urinary levels have been described yet.

3.2.6 Biomarkers for exposure to zearalenone

Malekinejad et al. (2006) showed that the biotransformation of ZEN is species dependent and there is a small amount of data related to human metabolism of this compound. It is generally known that there are two main metabolites occurring in animals and humans: α-ZEL and β-ZEL. Both metabolites undergo two further biotransformation pathways and can be conjugated with glucuronic acid. Glucuronidation is an important pathway in detoxification and elimination of ZEN, and in some species (e.g. pigs) ZEN undergoes total glucuronidation (Malekinejad et al., 2006). Most data regarding the presence of ZEN in physiological fluids and compartments are available as toxicokinetic studies in animal models. In rats and pigs, it was isolated from serum, urine, bile and faeces following oral and intravenous administration. In humans, ZEN and its metabolites α-ZEL and β-ZEL have been isolated from naturally contaminated urine samples (Solfrizzo et al., 2014), but there is no data correlating their presence in urine with the extent of ZEN exposure. Very often, ZEN derivates in urine were found to be conjugated with glucuronic acid, so it would be viable to evaluate glucuronides in humans as possible biomarkers for ZEN.

3.3 BIOMONITORING IN MYCOTOXIN RESEARCH: STATE OF THE ART

Biomarker research for human exposure assessment to mycotoxins started in the late 1980's when extensive studies on the carcinogenic aflatoxins were conducted (Wild and Turner, 2002). They have been essential for the establishment of the role of aflatoxins in human disease through better estimates of exposure, expanded knowledge of the mechanisms of disease pathogenesis, and as tools for implementing and evaluating preventive interventions. Later in the 1990's work on OTA (Duarte et al., 2011) and the fumonisins (Shephard et al., 2007) was conducted. The first biomarker research on DON was initiated by Meky et al. (2003).

As a result of the latest generation of high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) instruments, a clear trend towards the development and

application of multi-analyte methods in mycotoxin biomarker research can be observed. Purification of the analytes is often achieved by using extensive sample cleanup approaches (Ediage et al., 2012). However, the latest studies have also successfully applied the so-called dilute and shoot approach by omitting any cleanup step (Warth et al., 2012b). This chapter provides a short overview of published (multi)biomarker methods, discusses challenges associated with biomarker analysis and summarises human exposure assessment studies performed within the past decade.

3.3.1 Biomarker methods and their application in exposure assessment studies

Single biomarker methods

The first biomarker research on **DON** was initiated by Meky et al. (2003). This biomarker research investigated the metabolism of DON in rats as a basis to establish methodology for a candidate biomarker of human exposure and this methodology was tested on urine samples from a potentially highly exposed population. Urine samples were collected from female inhabitants of Linxian County, a high risk region for oesophageal cancer and Gejiu, a low risk region in China. DON was detected in all 15 samples following **β-glucuronidase** treatment and immunoaffinity column (IAC) enrichment with the identity of DON being confirmed by mass spectrometry (LOD 4 ng/mL). The mean levels of DON from the suspected high and low exposure regions of China were 37 ng/mL (14-94 ng/mL) and 12 ng/mL (4-18 ng/mL), respectively. Furthermore, Turner et al. (2008a) have developed a **LC-MS/MS** method with IAC enrichment and inclusion of ^{13}C-DON as an internal standard. The developed method was tested in a pilot survey performed in the UK whereby 25 healthy adults participated. Urine samples were analysed for DON following β-glucuronidase treatment (LOQ 0.6 ng/mL). This intervention study involved 2 days of normal diet and 4 days of a wheat-restricted diet. During consumption of the normal diet, all individuals had detectable urinary levels of DON, while fewer samples were positive (36 %) during the intervention. The geometric mean level during normal diet was 7.2 ng/mg creatinine (4.9-10.5 ng/mg creatinine). This is 11-fold higher than the 0.6 ng/mg creatinine (0.4-0.9 ng/mg

creatinine) during the intervention. This study found a significantly association between urinary DON and cereal intake. Additionally, Turner et al. (2010a) refined the previous method by adding **DOM-1** to the method (LOD 0.05 ng/mL). The refined method was applied to a pilot survey of male French farmers (n= 76, aged 23-74) whereby DON was detected in 75/76 urine samples (0.5-28.8 ng/mL) and DOM-1 in 26/76 samples (0.2-2.8 ng/mL). It was the first exposure biomarker survey for DON in a French population, and the first demonstration of urinary DOM-1 in humans. Hepworth et al. (2012) conducted a study in a subset of **pregnant women** from Bradford, UK. Women aged 16–44 years (n= 85) provided a urine sample for DON analysis in the last trimester of pregnancy, and completed a food frequency questionnaire (FFQ). The urinary DON biomarker was detected in all measured samples (0.5-116.7 ng/mg creatinine). Levels were higher in women classified as South Asian in origin compared with non-South Asians. This was the first biomarker demonstration of DON exposure in pregnant women. Srey et al. (2014) assessed DON exposure in children from three geographic locations within Tanzania, over three time points in 1 year using the same LC-MS/MS method. A total of 166 children aged 6–14 months were studied at maize harvest and followed up twice at 6-month intervals. On two consecutive days, morning urine was collected from each child and urinary DON was measured using an LC-MS/MS method, **with and without β-glucuronidase** hydrolysis in order to assess free DON and its glucuronides. Overall, urinary DON increased significantly along with the three visits. 51 % of the urine samples collected during the first visit were contaminated with DON (mean 1.1 ng/mL; 0.8-1.4 ng/mL), while 70 % was contaminated during visit two (mean 2.3 ng/mL; 1.7-3.2 ng/mL). 80 % of the samples was positive for DON at the third sample collection (mean 5.7 ng/mL; 2.34.1-7.9 ng/mL).

Warth et al. (2011) developed a LC-MS/MS method allowing quantification of both DON (LOD-LOQ 6-20 ng/mL) and **DON3GlcA** (LOD-LOQ 3-10 ng/mL) by a simple **dilute and shoot** approach without the need for any cleanup. The applicability of the method was demonstrated through the analysis of urine samples obtained from a volunteer during a regular and cereal-restricted diet. All four urine samples obtained from the cereal-restricted diet showed neither DON3GlcA nor DON contamination. In contrast, both samples analysed after normal diet including cereal products were positive for the glucuronide conjugate (31

and 32 µg/L). Furthermore, Warth et al. (2012a) performed a pilot survey to investigate the level of DON exposure in Austrian adults (n= 27) by measurements of DON (LOD-LOQ 4-13 ng/mL) and its glucuronides in first morning urine. The average concentration of total DON (22 % free + 96 % glucuronides) was estimated to be 20.4 µg/L (max. 63 µg/L) whereby one third of the volunteers exceeded the TDI when consuming regular diet. DON-glucuronides were **directly quantified by LC-MS/MS** and the results were compared with indirect quantification after enzymatic hydrolysis and confirmed the suitability of the direct method. **DON15GlcA** (LOD-LOQ 3.2-10.6 ng/mL) could be identified as a major DON metabolite in human urine based on the analysis of these samples. About 25 % of total DON glucuronides were derived from DON3GlcA (LOD-LOQ 6-20 ng/mL). Recently, Gratz et al. (2014) conducted a pilot study in the UK, whereby spot urine samples were collected in two consecutive years from 15 volunteers following their habitual diet. Urinary DON was analysed by LC-MS/MS after hydrolysis and IAC enrichment to estimate 24-h DON excretion and daily dietary DON intake. DON (LOD 0.10 ng/mL) was detectable in all urine samples with an average excretion of 10.08 µg/24h in year one, which significantly increased in year 2 (24.84 µg/24h). The TDI for DON was exceeded in 13 % of occasions in year 2 (mean intake 518.64 ng/kg BW/day) and none in year 1 (mean intake 195.94 ng/kg BW/day) suggesting that DON exposure varies annually.

The aim of the study performed by Munoz et al. (2010) was to develop a method that allows analysis of **OTA** (LOQ-LOQ 0.02-0.05 ng/mL) and its detoxication product **OTα** (LOQ-LOQ 0.02-0.05 ng/mL) in urine and in blood plasma. The method involves enzymatic hydrolysis of conjugates, liquid–liquid extraction, and analysis of sample extracts by high performance liquid chromatography with fluorescence detection (**HPLC-FD)**. Application of the validated method in a pilot study with 13 volunteers from Germany revealed the presence of OTA (0.02-0.13 ng/mL) and OTα (0.05-4.7 ng/mL) in all samples. Duarte et al. (2010) evaluated the exposure of the Portuguese population (n= 155) to OTA (LOQ 0.008 ng/mL). Morning urine samples were collected during the Winter of 2007, from each of five geographically distinct Portuguese locations and subjected to extraction by IAC before HPLC-FD analysis. 92.2 % of the urine samples were positive for OTA (mean 0.018 ng/mL; max. 0.069 ng/mL). Duarte et al. (2012) examined factors related to OTA exposure in the adult population of

Portugal over a one-year period. Anthropometric measures, season of the year and region were the selected factors correlated with OTA exposure biomarker. Urine samples from 95 inhabitants (50 women and 45 men) from six Portuguese main geographical areas were analysed using a HPLC–FD method (Pena et al., 2006) and included IAC enrichment (LOD-LOQ 0.008-0.024 ng/mL). Exposure to OTA proved to markedly increase in winter (87.4 %; mean 0.022 ng/mL), and gender differences were observed only in summer (81.1 %; mean 0.016 ng/mL), which might be related to different dietary patterns not only between seasons, but also between genders. Furthermore, Ali et al. (2014) collected 64 blood samples from healthy university students (32 female, 32 male) in Bangladesh for biomarker analysis. OTA (LOD-LOQ 0.05-10 ng/mL) and its metabolite OTα (LOD-LOQ 0.05-10 ng/mL) were determined in the plasma samples by a validated method using HPLC-FD analysis. After liquid–liquid extraction, OTA was detected in all plasma samples (0.20-6.63 ng/mL) and OTα was detected in 95 % of the samples (0.10-0.79 ng/mL). The OTA mean level in plasma of males and females were not significantly different. Statistical analysis of food consumption data for the participants, provided in a FFQ, did not reveal a significant association between OTA level in plasma and their intake of typical staple foods (rice, wheat, maize and lentil). The dietary intake of OTA calculated on the basis of plasma concentration in Bangladeshi students was lower than the TWI of OTA. Additionally, Munoz et al. (2014) conducted a longitudinally designed study in Chilean mother–child pairs (n= 21) with parallel collection of maternal blood, milk and infant urine samples over a period of 6 months. Validated LC-MS/MS methods were applied to determine OTA concentrations in all 134 biological samples (LOD-LOQ urine 30-50 ng/L). OTA was detected in almost all maternal blood plasma at concentrations ranging between 72 and 639 ng/L. Infants' exposure was calculated as daily intake from OTA levels in breast milk taking into account milk consumption and body weight. The statistical analysis showed a good correlation (r= 0.57) between OTA intake and infant urine concentrations (30-433 ng/L) during breastfeeding period and thereby confirms that urinary OTA analysis in infants is a valid biomarker of exposure.

Van der Westhuizen et al. (2011a) conducted the first study in South Africa assessing fumonisin exposure by evaluating Sa, So and Sa:So ratios in plasma and urine of participants as possible biomarkers of exposure. After HPLC-FD analysis of 41 plasma and 62 urine

samples no association was found between sphingoid base levels in the plasma and urine (1.9-40 ng/mL) and individual fumonisin exposure.

Blaszkewicz et al. (2013) developed a sensitive method for the analysis of **CIT and HO-CIT** in human blood and urine to investigate human exposure. Clean-up of blood plasma by protein precipitation followed by **LC–MS/MS** analysis allowed robust detection of CIT. For urine, sample clean-up by an IAC proved to be clearly superior to solid phase extraction (SPE) prior to LC–MS/MS analysis (LOQ-LOQ 0.02-0.05 ng/mL). The newly developed method was applied in a small pilot study. CIT was present in all plasma samples (n=8) at concentrations ranging from 0.16 to 0.79 ng/mL. CIT was detected in 8/10 urines (0.05-0.2 ng/mL) and HO-CIT (LOD-LOQ 0.05-010 ng/mL) was present in 5/10 urine samples (0.15-1.12 ng/mL). Ali et al. (2014) applied this method to investigate CIT exposure in 50 German adults (n= 27 females and n= 23 males). CIT and HO-CIT were detected in 82 % and 84 % of all urine samples, at concentrations ranging from 0.02 to 0.08 ng/mL for CIT, and 0.05 to 0.51 ng/mL for HO-CIT. Except for higher urinary CIT levels in males, differences between subgroups were not significant.

Multi-biomarker methods

The first method described for the determination of various mycotoxin biomarkers in human urine was developed by Ahn et al. (2010). To achieve sufficient sensitivity and selectivity, AFM_1, OTA, FB_1 and FB_2 were concentrated using three separate IAC's and OTα was extracted using liquid-liquid extraction. Also two other published multi-biomarker methods used the selectivity of antibodies by applying a multi-IAC column which comprises antibodies specific for aflatoxins, OTA, fumonisins, DON, ZEN, T-2 and HT-2 (Rubert et al., 2011; Solfrizzo et al., 2011a). Solfrizzo et al. (2011a) developed a **LC-MS/MS** method for simultaneous determination of AFM_1, OTA, DON, DOM-1, α-ZEL, β-ZEL and FB_1 in human and pig urine. Urine samples were purified and concentrated by a double cleanup approach, using a multi-toxin IAC and an Oasis® HLB column. Ten samples of human urine were

collected from healthy Italian volunteers (five male and five female) with a range of ages (26–87 years old). All samples were analysed twice, i.e. **without and with enzymatic digestion** with β-glucuronidase before sample cleanup, with increasing concentrations after digestion. Most of the samples tested positive for DON and/or OTA whereas no other biomarkers were detected. Co-occurrence of OTA and DON (70 %) in human urine was for the first time reported. The developed method was further used to detect levels of DON, DOM-1, AFM_1, FB_1, ZEN, α-ZEL, β-ZEL and OTA in urine samples of 52 volunteers resident in Apulia region in Southern Italy (Solfrizzo et al., 2014). The presence of ZEN + ZEL's, OTA, DON, FB_1 and AFM_1 was detected in 100 % (0.120 ng/mL + 0.176 ng/mL), 100 % (2.129 ng/mL), 96 % (67.36 ng/mL), 56 % (0.352 ng/mL) and 6 % (0.146 ng/mL) of the samples, respectively. Urinary biomarker concentrations were used to estimate human exposure to multiple mycotoxins. For OTA and DON, 94 % (0.053 µg/kg BW/day) and 40 % (1.03 µg/kg BW/day) of the volunteers exceeded TDI for these mycotoxins. A simple and rapid method for quantitative determination of DON, T-2, HT-2, ZEN, OTA, aflatoxins and fumonisins in urine was developed by (Rubert et al., 2011). For clean-up and extraction, the mixture was loaded on a IAC and samples were analysed using an ion trap mass spectrometer for detection. In a pilot study in the UK with 27 volunteers, OTA (11.1 %), DON (33.3 %) and AFG_2 (3.7 %) were detected (Rubert et al., 2011). A LC-MS/MS method developed by Ediage et al. (2013) covers seven mycotoxins and several important conjugation and breakdown products (in total 18 analytes). Sample cleanup was optimised in a progressive procedure where urine samples were extracted using liquid-liquid extraction followed by SPE. More detailed information about this method can be found in section 5.3.1. This study was conducted to investigate mycotoxin exposure in children (n= 220, aged 1.5–4.5 years) from high mycotoxin contamination regions of Cameroon and to examine the association between the mycotoxin levels (in total 18 analytes) and several socio-demographic factors and anthropometric characteristics. DON (0.1-77 ng/mL), OTA (0.04-2.4 ng/mL), AFM_1 (0.06-4.7 ng/mL), FB_1 (0.06-48 ng/mL), ZEN (0.65-5 ng/mL), α-ZEL (0.26-1.3 ng/mL) and β-ZEL were detected in respectively 17 %, 32 %, 14 %, 11 %, 4 %, 8% and 4 % of the urine samples. There were significant differences in the mean contamination levels of OTA and β-ZEL between the two agro-ecological zones investigated. Likewise significant differences were observed in the mean levels of AFM_1 across the weaning categories of these children. The mean

concentration of AFM_1 detected in the urine of the partially breastfed children was significantly higher than those of the fully weaned children. Meanwhile, the mean concentrations of DON and FB_1 detected in the urine of the male children was significantly different from the levels detected in the urine of female children. In this study, there was no association between the different malnutrition categories (stunted, wasting and underweight) and the mycotoxin concentrations detected in the urine of these children.

Warth et al. (2012b) developed an **LC-MS/MS** method for the quantitative measurement of 15 mycotoxins and key metabolites in human urine. DON, DON3GlcA, DON15GlcA, DOM-1, nivalenol (NIV), T-2, HT-2, ZEN, ZEN14GlcA, α- and β-ZEL, FB_1, FB_2, OTA and AFM_1 were determined without the need for any cleanup using a rapid and simple **dilute and shoot** approach. The applicability of the method was demonstrated by the analysis of urine samples obtained from Cameroon (n= 175). In naturally contaminated urine samples up to six biomarkers of exposure (AFM_1, DON, DON15GlcA, NIV, FB_1 and OTA) were detected simultaneously. The same LC-MS/MS method was used by Sarkanj et al. (2013) in a pilot survey conducted in pregnant women (n=40) from eastern Croatia. DON and its glucuronides were detected in 98 % of the studied samples, while OTA was found in 10 % (<4 ng/mL) of the samples. DON exposure was primarily reflected by the presence of DON15GlcA with a mean concentration of 120 ng/mL (1-1237.7 ng/mL), while free DON was detected with a mean concentration of 18.3 ng/mL (9-275 ng/mL). Several highly contaminated urine samples contained a third DON conjugate, tentatively identified as deoxynivalenol-7-glucuronide. 48 % (0.1-33.1 µg/kg BW/day) of subjects were estimated to exceed the TDI of DON. Furthermore, Ezekiel et al. (2014) performed a pilot, cross-sectional, correlational study in eight rural communities in northern Nigeria to investigate mycotoxin exposures in 120 volunteers (19 children, 20 adolescents and 81 adults) using the same LC–MS/MS based multi-biomarker approach. First morning urine samples were analysed and urinary biomarker levels were correlated with mycotoxin levels in foods consumed the day before urine collection. A total of eight analytes were detected in 50.8 % of the studied urine samples, with OTA (28 %; mean 0.2 ng/mL), AFM_1 (14 %; mean 0.3 ng/mL) and FB_1 (13 %; mean 4.6 ng/mL) being the most frequently occurring biomarkers of exposure. A pilot study was conducted whereby 60 first-morning urine samples were collected from healthy

volunteers who live in the Bangkok metropolitan area and surrounding provinces (Pathumthani, Nonthaburi, Samutprakarn and Samutsakorn). Urine samples were analysed using the same 'dilute and shoot' approach. AFM_1, OTA, DON3GlcA and DON15GlcA were detected in respectively 5 % (mean 0.16 ng/mL), 1.7 % (0.33 ng/mL), 5 % (mean 4.3 ng/mL) and 11 % (mean 8.1 ng/mL) of the individuals. The maximum concentrations were used to estimate the daily intake confirming that none of the individuals exceeded the TDI of DON or OTA. This was the first multi-mycotoxin biomarker study performed in South-East Asia (Warth et al., 2014). Abia et al. (2013) studied the frequency and level of exposure to multiple mycotoxins in human urine from Cameroonian adults. 175 urine samples (83 % from HIV-positive individuals) and a FFQ were collected from Cameroonians, and analysed for 15 mycotoxins and relevant metabolites using the LC-MS/MS method developed by Warth et al. (2012b). In total, eleven analytes were detected individually or in combinations in 110/175 (63 %) samples including the biomarkers AFM_1 (0.17-1.38 ng/mL), FB_1 (1.7-14.8 ng/mL), FB_2 (1.7 ng/mL), NIV (10-22 ng/mL), ZEN (1.3-1.42 ng/mL), ZEN14GlcA (3.3-31 ng/mL), OTA (0.17-1.87 ng/mL), DON (13 ng/mL), DON15GlcA (20-66.2 ng/mL) and DON3GlcA (20-22.8 ng/mL).

Huybrechts et al. (2014) developed a LC-MS/MS method for the detection of 32 biomarkers in urine samples without any sample clean-up. More detailed information about the method can be found in section 5.3.2. The method was pilot-tested in 32 Belgian volunteers whereby all urine samples contained DON15GlcA (3.1-420.1 ng/mL). Also DON3GlcA (0.5-55.4 ng/mL) and DOMGlcA (0.5-16.4 ng/mL) were present in, respectively, 90 and 25 % of the samples, while DON (0.5-3.1 ng/mL) was detected in 60 % of the samples, in lower concentrations. OTA (3-32.6 pg/mL) was detected in 70 % of the samples. CIT and HO-CIT were detected in 59 % (3-117 pg/mL) and 66 % (30-208.5 pg/mL) of the samples.

Rodriguez-Carrasco et al. (2014a) performed a pilot survey whereby human urine samples were analysed for presence of 15 mycotoxins and some of their metabolites using a novel urinary multi-mycotoxin gas chromatography tandem mass spectrometry (**GC–MS/MS)** method following salting-out liquid–liquid extraction. Fifty-four urine samples from children

and adults in Valencia were analysed for the presence of urinary mycotoxins. Four out of 15 mycotoxins were detected. 37 samples showed quantifiable values of mycotoxins. DON (mean-max. 25.1-84.5 µg/g creatinine) was the most frequently detected mycotoxin (68.5 %) whereby 8.1 % of total subjects were estimated to exceed the TDI. NIV (mean 15.13 µg/g creatinine) was found in 13 % of the samples, HT-2 (mean 13.67 µg/g creatinine) in 7.4 % and DOM-1 (mean 2.75 µg/g creatinine) in 3.7 %.

Shephard et al. (2013) applied novel urinary multi-mycotoxin **LC–MS/MS methods** to determine multiple exposure biomarkers in the high oesophageal cancer region, Transkei, South Africa. Fifty-three female participants donated part of their maize-based evening meal and first void morning urine, which was analysed both with sample clean-up (single and multi-biomarker) and by a 'dilute-and-shoot' multi-biomarker method. A single biomarker method detected FB_1 (LOD-LOQ 0.01-0.02 ng/mL) in 87 % of the urine samples with a concentration between 0.01-1.3 ng/mL (Gong et al., 2008). After hydrolysis with β-glucuronidase was DON (LOD-LOQ 0.25-0.50 ng/mL) detected in 100 % of the samples between 0.3-99.2 ng/mL (Gong et al., 2008; Turner et al., 2008a). The multi-biomarker 'dilute-and-shoot' method indicated the presence of DON15GlcA in 55 % of the samples with a maximum concentration of 47 ng/mL (Warth et al., 2012b). A multi-biomarker method with β-glucuronidase and IAC clean-up determined ZEN (100 %; 0.012-3.15 ng/mL), FB_1 (96 %; 0.04-4.94 ng/mL), α-ZEL (92 %; 0.009-3.72 ng/mL), DON (87 %; 0.45-53.4 ng/mL), β-ZEL (75 %; 0.016-5.94 ng/mL) and OTA (98 %; 0.002-0.432 ng/mL) (Solfrizzo et al., 2011b). All the LOD and LOQ values of the different multi-biomarker methods mentioned, are summarized in table 3.2.

Table 3.2. LOD and LOQ values of the different multi-biomarker methods

LOD/LOQ ng/mL	Ahn et al. (2010)	Solfrizzo et al. (2011a)	Rubert et al. (2011)	Warth et al. (2012b)	Rodriguez-Carrasco et al. (2014a)
AFM_1	0.003/0.010	0.020/-	-	0.05/0.17	-
OTA	0.001/0.004	0.006/-	0.5/1.5	0.05/0.17	-
FB_1	0.007/0.022	0.010/-	5/15	0.5/1.7	-
FB_2	0.003/0.010	-	4/15	0.5/1.7	-
OTα	0.045/0.135	-	-	-	-
DON	-	1.5/-	10/35	4/13	0.12/0.25
DON3GlcA	-	-	-	6/20	-
DON15GlcA	-	-	-	6/20	-
DOM-1	-	9.9/-	-	10/33	0.25/0.50
NIV	-	-	-	3/10	0.5/1
ZEN	-	0.007/-	3/10	0.4/1.3	3/6
ZEN14GlcA	-	-	-	1/3.3	-
α-ZEL	-	0.030/-	-	0.5/1.7	1/2
β-ZEL	-	0.054/-	-	0.5/1.7	2/4
T-2	-	-	2/6	2/6.7	0.5/1
HT-2	-	-	3/10	20/67	1/2
AFB_1	-	-	0.5/1.5	-	-
AFB_2	-	-	0.4/1.2	-	-
AFG_1	-	-	0.4/1.2	-	-
AFG_2	-	-	0.8/2	-	-
3ADON	-	-	-	-	0.25/0.50
FusX	-	-	-	-	2/4
DAS	-	-	-	-	1/2
NEO	-	-	-	-	0.25/0.50
ZAN	-	-	-	-	4/8
α-ZAL	-	-	-	-	4/8
β-ZAL	-	-	-	-	4/8

**ZAN = zearalanone ; α-ZAL = α-zearalanol;* β*-ZAL =* β*-zearalanol*

3.3.2 Analytical challenges

A major challenge in mycotoxin biomarker research is the extremely low analyte concentrations present in biological fluids following dietary exposure. Hence, appropriate sample preparation protocols are crucial to obtain acceptable LOD's. This is, however, challenging due to the chemical diversity of analytes typically included in multi-biomarker methods. This issue becomes even more complex once polar conjugates such as glucuronides are included as they are frequently lost during common cleanup approaches such as SPE or IAC procedures (Warth et al., 2012b). The great advantage of the methods using IAC's for cleanup is the specific retention of the target compounds only. Thereby, potentially interfering matrix compounds are removed efficiently. The major disadvantage is that no conjugates or other biomarkers of interest can be included in a method. Furthermore, enzymatic hydrolysis should be performed to include conjugates, and the overall procedure is time-consuming and costly and requires a labor-intensive sample preparation. This is in contrast to the dilute and shoot approach, where a urine sample is centrifuged, diluted, and analysed without further pretreatment. However, to overcome matrix effects and interfering matrix peaks, variables such as eluents and the chromatographic gradient need to be carefully optimised (Warth et al., 2012b; Warth et al., 2013b).

Ediage et al. (2012) investigated different procedures including dilute and shoot, dilute, evaporate, and shoot, liquid–liquid extraction, and concluded that the approaches based on sample dilution yielded unfeasibly high LOD's and significant signal enhancement for ZEN and FB_1. Furthermore, Warth et al. (2012b) tested various SPE cartridges during method development of the dilute and shoot method. Most of these SPE columns failed to retain the polar glucuronide conjugates, with the exception of the Oasis® HLB. When using dilute and shoot methods, co-eluting matrix components can influence the accuracy of quantitative methods through ion suppression or enhancement. These matrix effects can be controlled by using matrix-matched calibration (Solfrizzo et al., 2011a), inclusion of ^{13}C labelled internal

standards (Ahn et al., 2010; Turner et al., 2008b), or correction of results with the apparent recovery (Huybrechts et al., 2014).

In the past, most biomarker methods focused on parent mycotoxins rather than on conjugated forms as no (certified) calibrants are commercially available for these metabolites. Furthermore, direct quantification of mycotoxin conjugates without enzymatic hydrolysis is commonly used as incomplete hydrolysis and the time-consuming sample preparation can be overcome. An important quality control measure is the use of certified reference materials including well-characterized calibrants to monitor the performance of a certain method. However, for mycotoxin biomarkers, there is no matrix reference material available that would make it possible to assess the measurement performance in the analysis of biologically important matrices. This is critical especially in view of the complex biological matrices and makes efforts such as a recent interlaboratory comparison (Solfrizzo et al., 2013) even more important to ensure analytical accuracy.

CHAPTER 4

AIMS AND RESEARCH QUESTIONS

Mycotoxins are secondary metabolites produced by fungi. These naturally occurring toxins are important harmful food contaminants responsible for health effects such as cancer, nephrotoxicity, hepatotoxicity or immunosuppression. More than 400 mycotoxins have been reported, whereby AFB_1, DON, FB_1, OTA, ZEN and T-2 form the greatest risk for human and animal health (Riley and Pestka, 2005). People can be exposed through consumption of contaminated food, dermal contact and inhalation of toxins present in dust and air (Miraglia et al., 2009). Although the European Commission established maximum levels for these mycotoxins in food and feed, mycotoxins form a worldwide problem (1881/2006/EC; 2006/576/EC).

In order to study the impact of mycotoxins on the public health, it is important to assess human exposure. The assessment of mycotoxin exposure is mainly based on calculations combining mycotoxin occurrence data in food with population data on food consumption (De Boevre et al., 2013; Gauchi and Leblanc, 2002; Kuiper-Goodman et al., 2010). The accuracy of that approach is limited due to the heterogeneous distribution of mycotoxins in food, the possible exposure through inhalation, the presence of masked mycotoxins, the influence of food processing, inter-individual variation in ADME processes and the under- and overestimation in food consumption data (Arcella and Leclercq, 2004). In order to improve and refine risk assessments, human biomonitoring has become an added value in evaluating exposure to mycotoxins. The direct measurement of biomarkers of exposure is the only available tool that integrates exposures from all sources. Biomarkers of the most common mycotoxins have been validated in biological fluids such as urine. The individual variation in ADME processes is integrated when using biomarkers, whereby a more accurate assessment of exposure can be performed at the individual level. Furthermore, human biomonitoring can demonstrate trends and changes in exposure, establish distribution of

exposure, identify vulnerable groups and it can reduce the assumptions regarding consumption rates. For this reason, the combination of exposure data from both methods will lead to a better interpretation in risk assessment (Choi et al., 2015). Only recently (past 10 years), results of epidemiological studies were increasingly reported whereby the exposure of mycotoxins in different populations was assessed through biomarkers (Duarte et al., 2012; Ediage et al., 2013; Gilbert et al., 2001; Pena et al., 2006; Rubert et al., 2011; Turner et al., 2010a; Van der Westhuizen et al., 2011b). Exposure assessment of mycotoxins using biomarkers in the Belgian population was before this doctoral research (based on the BIOMYCO study) still missing.

The BIOMYCO study was designed to assess human mycotoxin exposure based on the direct measurement of urinary biomarkers using validated LC-MS/MS methods. Over the different seasons in 2013 and 2014, morning urine was gathered in a representative part of the Belgian population according to a designed study protocol (chapter 5). In first instance, different mycotoxins and their metabolites were measured in the urine samples and mycotoxin exposure was described for the child and adult group (chapter 6). Second, mycotoxin exposure of different subgroups (age, gender, …) was compared to test whether exposure was significantly different between subgroups of the population. Third, links between the mycotoxin concentrations measured and the food consumption reported were estimated, to explore whether the mycotoxin exposure could be explained by the consumption of certain foods (chapter 7). Finally, a risk assessment was performed by comparing the estimated intakes with the TDI's (chapter 6). The results of this study can be of interest for policy makers to get new insights in the mycotoxin issue and to help them in the decision-making process for the prevention and control of mycotoxin exposure.

PART2

Assessment of mycotoxin exposure in the Belgian population

Adapted from

Ellen Heyndrickx, Isabelle Sioen, Mia Bellemans, Mieke De Maeyer, Alfons Callebaut, Stefaan De Henauw and Sarah De Saeger (2014). **Assessment of mycotoxin exposure in the Belgian population using biomarkers: Aim, design and methods of the BIOMYCO study.** *Food Additives & Contaminants: Part A, Vol. 31, No. 5, 924–931.*

Ellen Heyndrickx, Isabelle Sioen, Bart Huybrechts, Alfons Callebaut, Stefaan De Henauw and Sarah De Saeger (2015). **Human biomonitoring of multiple mycotoxins in the Belgian population: Results of the BIOMYCO study.** *Environment International (in press).*

Ellen Heyndrickx, Isabelle Sioen, Bart Huybrechts, Mia Bellemans, Mieke De Maeyer, Alfons Callebaut, Stefaan De Henauw and Sarah De Saeger (2015). **Urinary mycotoxin biomarkers in relation to food consumption and socio-demographical characteristics in Belgian children and adults.** *In preparation.*

CHAPTER 5

DESIGN AND METHODS OF THE BIOMYCO STUDY

5.1 STUDY DESIGN

The BIOMYCO study was conducted according to the guidelines laid down in the declaration of Helsinki and was approved by the Ethical Committee of the Ghent University Hospital. All participants needed to sign an informed consent, whereby the informed consent for participating children was signed by their parents whereas a verbal assent was obtained from the children. Each participant was informed about their right to withdraw from the study at any time without any reason. Figure 5.1. schematically presents the study design of the project with the corresponding measurements. It is important to note that no medical examinations were carried out in this study and volunteers were not restricted to any diet.

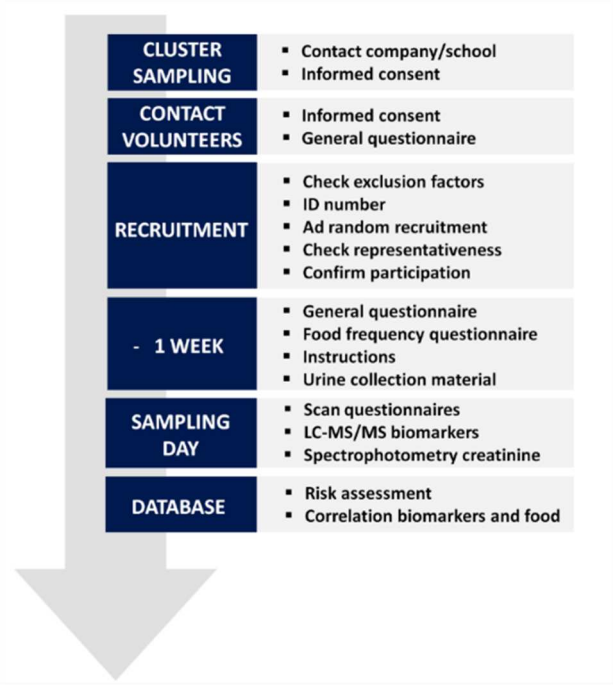


Figure 5.1. Flow diagram of the study design

5.1.1 Target population and sample size

A sample size calculation was performed to determine the sample size needed to assess the exposure to mycotoxins in the target population. Because of the significant DON occurrence in Belgium (De Boevre et al., 2013), the sample size calculation for adults (between 19 and 65 years old) was performed using results of an exposure assessment of DON done in the UK (Turner et al., 2010a). With a variance in DON concentration of 0.85, a confidence interval of 95 % and an error of 1.10, the number of adults to be studied was estimated at 278 in order to get a representative view of the mycotoxin exposure in Belgium.

Furthermore, young children need special attention in contaminant exposure assessment studies because of the quantitative higher food intake per kg body weight. For this reason, children within the defined age group of 3-12 years old were also recruited in this study. Children younger than 3 years could not participate because of logistic reasons for the collection of morning urine. A sample size calculation for children could not be performed because of the lack of information on the mean exposure to mycotoxins in this age group before the start of this study. We decided to recruit 140 children (being half of the sample size calculated for the adult group).

Table 5.1. The representative distribution based on statistical data of Belgium in 2011 (Belgian Federal Government, 2011)

ADULTS			
Age	**19-34 years**	**35-50 years**	**51-65 years**
	32 %	37 %	31 %
Region	**Flanders**	**Wallonia**	**Brussels**
	60 %	32 %	8 %
Gender	**Male**	**Female**	
	50 %	50 %	
CHILDREN			
Age	**3-6 years**	**7-9 years**	**10-12 years**
	40 %	30 %	30 %
Region	**Flanders**	**Wallonia**	**Brussels**
	56 %	35 %	9 %
Gender	**Male**	**Female**	
	51 %	49 %	

5.1.2 Timeframe

During the different seasons of 2013 and 2014, morning urine of adults and children was gathered across Belgium. Hereby, attempts were done to have a representative distribution for sex (male and female), age (3-6; 7-9; 10-12 and 19-34; 35-50; 51-65 years old) and geographical areas (Flanders, Wallonia and Brussels) within Belgium. The representative distribution was based on statistical data of Belgium in 2011 (Belgian Federal Government, 2011) and can be found in table 5.1. The collection of the urine samples and the food questionnaires was spread over the different seasons to be able to see whether there was a seasonal variation. Additionally, adults recruited in the first season were asked to participate a second time one year later in order to investigate the year variation in their mycotoxin exposure.

5.1.3 Cluster sampling

To enable the collection of the predefined number of samples, a random cluster sampling was carried out. Recruitment of participants at a school and company setting for respectively children and adults was performed, since random sampling of Belgian adults and children was not realistic given the timeline and budget available in the BIOMYCO project.

The companies and schools were selected by convenience based on the geographical location at first instance. Second, companies and schools were selected taking into account the variance in age and gender. The companies varied in size and served different sectors and training levels. Companies and schools were recruited from August 2012 onwards. The first step in contacting companies and schools consisted of sending an invitation letter to the director/principal of the company/school. Afterwards a researcher presented the study during a personal contact, telephone call, or provided written information about the study in order to explain aims and procedures. Each company/school needed to confirm its agreement by signing the informed consent, participated on a voluntary basis and gave

permission to invite their employees or children/parents to participate in the BIOMYCO study.

5.1.4 Call for participants

Participants were contacted providing an invitation letter, wherein they were informed and invited to participate or let their children participate in the BIOMYCO study. People could register for the study within two weeks after the call by signing the informed consent and by filling in a short questionnaire to collect some socio-demographical information. This information was used to control the representative distribution and could also be electronically collected by means of a web-based survey. All participants received a copy of the signed consent form.

5.1.5 Recruitment strategy

After registration, all general questionnaires were dealt with in the order they came in. In first instance, the exclusion criteria were checked: first, only one member per family could sign in; second, people who were exposed to a large amount of mycotoxins in another way than food such as farmers or veterinarians were excluded; third, persons with severe problems with liver, bile or kidney could not participate due to the related risk for interferences with the metabolism of mycotoxins and creatinine. The use of medication was not an exclusion criterion since there is limited information about possible interferences with mycotoxins. All data concerning medication was gathered in order to explain any possible deviant values afterwards.

When people met the inclusion criteria they received an identification number in order to process the information anonymous. The day on which the collection would take place, was assigned to each participant based on representativeness (sex, age, region, education level and diet) and logistic organisation.

5.1.6 Sample collection

All eligible participants received one week before the urine collection, a general questionnaire, a FFQ and a urine jar with instructions. These instructions contained clear information about how and when they had to fill in the questionnaires, when they had to collect their morning urine and where and when they could deliver sample and filled questionnaires. In agreement with the director/principal of the company/school, a room was reserved where the participants could hand in their questionnaires and morning urine to our fieldworkers. To minimise missing data, the fieldworkers immediately checked the completeness of questionnaires. Sample collection generally took place between 8-10 a.m.

5.1.7 Transportation and storage conditions

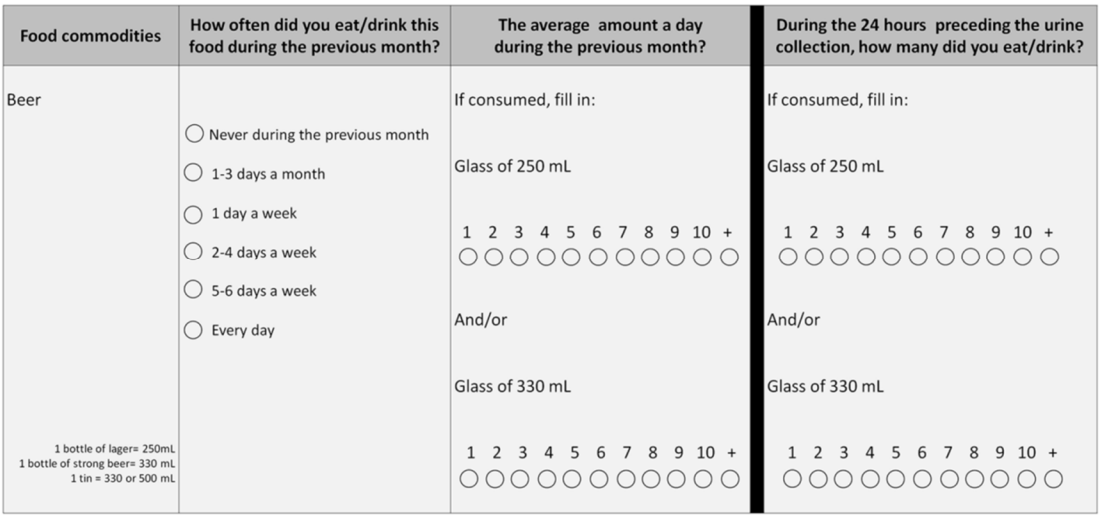
The sampling day was randomLy selected to cover all days of the week except for Saturday and Sunday. All urine samples were transported to the laboratory under cooling conditions, were divided in aliquots (20 mL) and stored at -20°C within six hours after urine collection for stability reasons (Ediage et al., 2012). All samples were registered in a sample book.

5.2 QUESTIONNAIRES

A general questionnaire was drawn up in order to collect personal and socio-demographical data for checking the representativeness of the population and to investigate whether mycotoxin exposure of different subgroups (age, …) was significantly different. Participants were asked for their medical history, smoking behaviour, recent medication, diet, dietary restrictions and socio-demographical data. Height and weight were self-reported to obtain individuals' body mass index (BMI). For the children, age and sex-specific BMI z-scores were calculated based on the reported weight and height using Flemish 2004 growth reference data (Roelants et al., 2009).

Because of the considerable differences between the toxicokinetics of the different mycotoxins in human and animals (see chapter 2), a FFQ was drawn up whereby the consumption of some foods in the 24 hours and month prior to the urine collection was asked. This 43 food-item containing instrument was developed and pilot tested by experienced dieticians and was used as a screening instrument to investigate the food consumption in adults and children. The participants were asked to report the frequency of consumption of selected food items using following response options: never, 1-3 days a month, 1 day a week, 2-4 days a week, 5-6 days a week and every day. Foods were not weighed, but amounts of consumption were assessed using household measures such as glass, cup, spoon or slices (figure 5.2.).

Figure 5.2. Example of the food frequency questionnaire, whereby the consumption of 43 food commodities in the 24 hours and month prior to the urine collection was asked



In addition the weights of these specific food items were estimated and the intake was determined of each food commodity (g/day or mL/day) for each individual. In order to calculate the intake levels during the previous month (g/day or mL/day), a conversion factor was used whereby the assumption was made that every month consists of 4 weeks. To prevent time-consuming questionnaires, only the consumption of food commodities that are suspected to be contaminated with mycotoxins and are highly consumed in Belgium were included in the questionnaire such as breakfast cereals, bread and bakery products, rice,

pasta, soy products, nuts and raisins (see table 5.2.). Other potential sources of mycotoxins do occur in the diet, such as in sauces and thickeners. However, their contribution to mycotoxin intake was estimated to be of relatively minor importance.

The questionnaires were made in Dutch and French using Teleform (‘*Cardiff Software’* Hewlett Packard, Illinois, USA) allowing electronic scanning and therefore minimising typing errors. Most importantly, all questionnaires were self-administered by the participants at home without interference of the researchers, whereby parents could fill in the questionnaires of their children. The questionnaire was accompanied by an instruction sheet, containing clear information about how and when they had to fill in the questionnaires. The questionnaires are in the annex of this thesis.

Table 5.2. The 43 food commodities studied in the food frequency questionnaire

Breakfast cereals	**Rusk**	**Soy products**
Muesli and cruesli	**Pasta**	Pudding - desserts
Cornflakes, rice krispies…	Wholemeal	Tofu
Pastry and biscuits	White	Tempeh
Cereal bar	Polenta	**Vegetarian burger**
Ginger bread	**Rice**	**Pizza/Durum**
Biscuits	Whole grain	**Tortilla**
Pancake	White	**Drinks**
Cake	**Nuts and dried fruit**	Coffee
Pastry and pie	Assorted nuts with raisins	Beer
Bread	Peanuts and peanut cheese	Wine
White	Pistachio nuts	Soy
Wholemeal	Other nuts	**Other**
Rye	Dried fruit (fig, apricot…)	Popcorn
Raisin	Raisins	Corn-based crisps
Soft white bread	**Spices**	Seitan
(Soft buns, brioche bread,…)	Paprika powder	Quorn
Viennoiseries	Chilli powder	Maize

5.3 ANALYTICAL METHODS

In most of the mycotoxin exposure studies with urinary biomarkers performed before the start of this project, urinary glucuronides were measured after enzymatic digestion with β-glucuronidase (Turner et al., 2010a; Turner et al., 2008c). The interferences of mucopolysaccharides with β-glucuronidase could lead to an inefficient hydrolysis and possibly inaccurate results (Orti et al., 1986; Pena et al., 2006). In this project, biomarkers such as the glucuronides were determined in a direct way. Therefore, two previously described LC-MS/MS methods were further optimised and validated for the determination of mycotoxin biomarkers in morning urine. In total 33 mycotoxins/metabolites were analysed in this project (table 5.3.).

Table 5.3. Mycotoxins and metabolites analysed in urine samples

Group	Mycotoxins and metabolites
Aflatoxins	AFB_1 AFB_2 AFG_1 AFG_2 AFM_1
Citrinin	CIT HO-CIT
Fumonisins	FB_1 FB_2 FB_3 HFB_1
Ochratoxins	OTA - OTα
Trichothecenes	T-2 HT-2 DON DON3GlcA DON15GlcA DOM-1 DOMGlcA
	3ADON 3ADON15GlcA 15ADON 15ADON3GlcA DAS FusX
Zearalenones	ZEN ZEN14GlcA α-ZEL α-ZEL7GlcA α-ZEL14GlcA β-ZEL β-ZEL14GlcA

5.3.1 LC-MS/MS method using sample clean-up

The first method was the LC-MS/MS method using sample clean-up. This LC-MS/MS method can analyse 13 potential biomarkers (mycotoxins and metabolites) within one chromatographic run of 28 min according to Ediage et al. (2012) with some minor modifications. Within the BIOMYCO project following biomarkers were analysed: AFB_1, AFM_1, DON, DOM-1, FB_1, HFB_1, HT-2, OTA, Otα, T-2, ZEN, α-ZEL and β-ZEL.

Mycotoxin reference standards namely AFB_1, HT-2, OTA, ZEN, FB_1, DOM, α-ZEL and β-ZEL, were purchased from Sigma-Aldrich (Bornem, Belgium). T-2 toxin was purchased from Biopure (Tulln, Austria). DON and AFM_1 were purchased from Fermentek (Jerusalem, Israel). OTα was obtained from Coring System Diagnostix (Gernsheim, Germany). Stock solutions of DON, AFM_1, AFB_1, HT-2, T-2, OTA, ZEN, FB_1, α-ZEL and β-ZEL were prepared in methanol at a concentration of 1 mg/mL. DOM and OTα were obtained as solutions in acetonitrile, with a concentration of 100 µg/mL and 10.3 µg/mL respectively. HFB_1 was synthesised by alkaline hydrolysis as described in Pagliuca et al. (2005). Purification of HFB_1 was carried out on a Waters HPLC instrument coupled to a Waters Fraction Collector III (Waters, Zellik, Belgium). Characterisation of the synthesised compound was performed by accurate mass measurements using an Exactive Orbitrap mass spectrometer (Thermo Scientific, Rockwood, USA). Quantification was carried out by spectrophotometric measurements as described in the original protocols. All stock solutions were stored at -20°C. From the individual stock solutions, a standard mixture was prepared in methanol, stored at -20°C and renewed every 2 weeks.

The optimum conditions for sample preparation were obtained using an experimental design (MODDE 9.0 software, Umetrics, Malmo, Sweden). In brief, mycotoxin biomarkers were extracted from 5 mL urine adding 10 mL ethyl acetate/formic acid (99/1, v/v) followed by extraction on an orbital shaker for 20 min. Centrifugation was later performed at 4 000 x *g* for 5 min. The ethyl acetate phase was dried at 40°C under a gentle stream of nitrogen. The pH of the aqueous phase was adjusted to pH between 6.5 and 7 with sodium carbonate (0.4 M) and further diluted with methanol (1/5, v/v). The biomarkers were further isolated by SPE based on anion exchange. The SPE cartridge was conditioned by passing 10 mL methanol/water (85/15, v/v) followed by 10 mL methanol. The loaded sample was allowed to flow-through at a flow rate of one drop per second. Afterwards the cartridge was washed with 1 mL water. The analytes were eluted with 5 mL methanol/formic acid (99/1, v/v). The eluate was combined with the residue obtained after extraction and the pooled extract was evaporated at 40°C. The residue of the combined fractions was reconstituted in 200 µL injection solvent which consisted of water/methanol/formic acid (61.8/37.9/0.3, v/v/v). Hexane (500 µL) was added and the mixture was vortexed for 1 min. The content was

brought into a centrifugal filter and after centrifugation for 5 min at 14 000 *x g*, a 150 µL aliquot of the aqueous phase was transferred to a vial for LC-MS/MS analysis. Isotope labelled internal standards for AFB_1, DON, FB_1, OTA and ZEN were added before LC-MS/MS analysis.

Detection and quantification were performed with a Waters Acquity UPLC® system coupled to a Micromass Quattro Micro triple quadruple spectrometer (Waters, Milford, MA, USA). The mass spectrometer analyses were carried out using multiple reaction monitoring mode and ionisation was performed in the positive electrospray ionisation (ESI) mode. The analytical column used was an Agilent ZORBAX SB-C18 (100 mm × 2.1 mm i.d., 3.5 µm). Two solvent mixtures were used as mobile phases, both containing 5 mM ammonium formate. Solvent A consisted of water/formic acid (99.7/0.3, v/v) while methanol/water/formic acid (94.7/5/0.3, v/v/v) was used as solvent B. The sample injection volume was 20 µL. A solvent gradient (flow rate of 0.25 mL min−1) was adopted for a total run time of 28 min. The solvent gradient was as follows: 0-1 min, 90 % A; 1-5 min, 90-50 % A, 5-10 min, 50-35 % A; 10-15 min, 35-20 % A; 15-25 min, 20-90 % A; 25-28 min, 90 % A. The primary transition of the most abundant product ion was used for quantification while the secondary transition was used for confirmation. Quantification was carried out with matrix-matched calibration curve whereby blank urine samples were used.

This method was validated following the guidelines of Commission Decision 2002/657/EC and Commission Regulation 401/2006/EC whereby LOD, limit of quantification (LOQ), apparent recovery, precision, selectivity/specificity and linearity were determined using blank urine samples. The lack of fit test for linearity resulted in p-value > 0.05 for all the analytes which illustrates the reliability of the chosen calibration range(s) for the quantification of the different analytes in real samples. In addition, the coefficient of determination (R^2) was also determined and was between 0.9774-0.9994 for all the different analytes. The apparent recovery data evaluated at concentrations of 10 times the method LOQ for the different analytes were expressed as bias. The values ranged from 1-15 % for all the compounds. The intra-day and inter-day precision values were in the range 5-19 % and

8-29 % respectively. The calibration range, LOD and LOQ for each analyte is shown in table 5.4. Other instrumental parameters and detailed information about this method can be found in Ediage et al. (2012).

Table 5.4. Concentration range, LOD and LOQ of the different analytes

Analytes	Concentration range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
DON	5-50	2.85	5.70
DOM	5-100	0.65	1.30
AFM_1	0.05-10	0.01	0.02
OTα	0.1-20	0.03	0.06
AFB_1	2-20	0.83	1.66
HFB_1	1-50	0.51	1.02
HT-2	1-20	0.42	0.84
β-ZEL	5-20	1.10	2.20
FB_1	0.2-20	0.05	0.10
T-2	0.2-20	0.05	0.10
α-ZEL	2-20	0.61	1.22
ZEN	5-50	1.24	2.48
OTA	0.1-10	0.03	0.06

5.3.2 LC-MS/MS method without sample clean-up

The second method was the LC-MS/MS method without sample clean-up. This LC-MS/MS method can analyse 32 potential biomarkers (mycotoxins and metabolites) within two chromatographic runs of each 30 min. Within the BIOMYCO project following biomarkers were analysed: AFB_1, AFM_1, AFB_2, AFG_1, AFG_2, CIT, HO-CIT, DAS, DON, DON3GlcA, DON15GlcA, 3ADON, 3ADON-15-glucuronide (3ADON15GlcA), 15ADON, 15ADON-3-glucuronide (15ADON3GlcA), DOM-1, DOMGlcA, FB_1, FB_2, FB_3, FusX, HT-2, OTA, OTα, T-2,

ZEN, ZEN14GlcA, α-ZEL, α-ZEL-7-glucuronide (α-ZEL7GlcA), α-ZEL-14-glucuronide (α-ZEL14GlcA), β-ZEL and β-ZEL-14-glucuronide (β-ZEL14GlcA).

Standards of FB_1, FB_2 and FB_3 were purchased from Cfm Oskar Tropitzsch (Marktredwitz, Germany) in powder form. DOM-1, AFB_1, AFB_2, AFG_1, AFG_2, DON, OTA, ZEN and T-2 were purchased in powder form Romer Labs (Tulln, Austria). 3ADON, 15ADON, α-ZEL, β-ZEL and HT-2 were purchased from Sigma-Aldrich (Bornem, Belgium). These were dissolved in pure acetonitrile or in a mixture of acetonitrile/water (1/1 v/v). HO-CIT was purchased from AnalytiCon Discovery GmbH (Potsdam, Germany) and dissolved in acetonitrile. AFM_1, DAS, CIT, OTα and FusX were purchased from Romer Labs as certified calibrant solutions. These solutions were stored at −20 °C. DON3GlcA was synthesised as described in Versilovskis et al. (2012) and the structure was confirmed from nuclear magnetic resonance analysis. DON3GlcA was quantified using UV-spectroscopy and confirmed by liquid chromatography-diode array detection. The synthesis and structure confirmation of DON15GlcA was described in Uhlig et al. (2013). The structures of all other glucuronides (DOMGlcA, ZEN14GlcA, β-ZEL14GlcA, α-ZEL7GlcA, α-ZEL14GlcA, 15ADON3GlcA, 3ADON15GlcA) were tentatively identified as described in Versilovskis et al. (2012). The concentration of DON15GlcA was determined based on the calibration curve using DON3GlcA. All other glucuronides were quantified semi-quantitatively, as not enough pure compounds were available for weighing.

Prior to the start of analysis all urine samples were centrifuged at 10 000 rpm for 5 min. A 2 mL portion was filtered with a syringe filter (0.2 µm). Detection and quantification were performed with a Waters Acquity UPLC® H-Class system coupled to a Xevo TQ-S spectrometer (Waters, Milford, MA, USA). The mass spectrometer analyses were carried out using multiple reaction monitoring mode and ionization was performed in the positive and negative ESI mode. The analytical column used was a Waters HSS T3 (100 mm x 2.1 mm i.d.; 1.8 µm). Water and methanol were used as mobile phases, both containing 5 mM ammonium acetate and 0.05 % acetic acid in positive ESI mode and 0.1 % acetic acid in negative ESI mode. The sample injection volume was 10 µL. A solvent gradient (flow rate of 0.5 mL min−1) was adopted for a total run time of 30 min. The gradient program started at 2.5 % methanol; after a plateau for 2 min at initial conditions, it increased to subsequently 4 %

in 6 min, 10 % at 10 min and reached 75 % at 27 min. The column was then washed with 99 % methanol for 1 min and equilibrated at initial conditions for 2 min. Quantification was carried out using an external calibration curve and correction for matrix suppression or enhancement.

Table 5.5. Concentration range, LOD and LOQ of the different analytes

Analytes	Concentration range (ng/mL)	LOD (pg/mL)	LOQ (pg/mL)
DON/DON3GlcA/DON15GlcA/DOMGlcA	0.5-20.0	200	500
FusX/β-ZEL/ZEN14GlcA/HT-2	0.5-20.0	200	500
3ADON15GlcA/15ADON3GlcA	0.5-20.0	50	200
OTα/α-ZEL7GlcA/β-ZEL/α-ZEL	0.5-20.0	50	200
FB_1/FB_3/ FB_2	0.5-20.0	50	200
DOM-1/3ADON/15ADON/α-ZEL14GlcA	0.5-20.0	100	300
HO-CIT/ T-2	0.5-20.0	10	30
AFG_2	0.05-2	10	30
AFM_1	0.05-2	2	5
AFG_1/ AFB_2/ AFB_1	0.05-2	5	20
DAS	0.5-20.0	5	20
CIT	0.5-20.0	1	3
OTA	0.05-2	1	3
ZEN	0.5-20.0	20	50

This method was validated for most of the 32 biomarkers following the guidelines of Commission Decision 2002/657/EC and Commission Regulation 401/2006/EC whereby LOD, LOQ, reproducibility, repeatability and linearity were determined using blank urine samples. Calibration curves generated R^2 values of at least 0.95. The RSD_{Rw} values were less then 20 % for 25 analytes, 23 % for DON, and between 31 and 41 % for DOM-1, 3ADON15GlcA, 15ADON3GlcA and β-ZEL14GlcA. The calibration range, LOD and LOQ for each analyte is shown in table 5.5. More detailed information about this method can be found in (Huybrechts et al., 2014).

5.3.3 Creatinine determination with spectrophotometry

The analysis of urinary mycotoxin biomarkers excretion in 24 hours samples is an accurate method to assess exposure. However, the collection of 24 hours urine is very aggravating for the participants, difficult for children and time-consuming. Therefore morning urine was used as alternative in order to prevent the drop out of participants, whereby the obtained biomarker concentrations were normalized in function of the creatinine content to correct for variable dilutions (Turner et al., 2010a). The urinary creatinine level was measured through an in-house spectrophotometric method based on the principle of Jaffe's reaction. Briefly, 18 mM picric acid reacts with 85 mM sodium hydroxide to form alkaline picrate. Alkaline picrate (2 mL) reacts with 1 mL of diluted urine (1/100, v/v, in ultrapure water). The optical density was measured at 495 nm after 25 min using a Philips PU 8620 spectrophotometer (Ediage et al., 2012). Furthermore, creatinine concentrations were used to determine whether the spot urinary sample was valid. The World Health Organization (WHO) recommends that if a sample is too diluted (creatinine concentration <30 mg/dL) or too concentrated (creatinine concentration >300 mg/dL), another urine void should be collected (WHO, 1996). For this reason, 42 urine samples with creatinine concentrations out of this range were excluded from the project.

Urinary creatinine concentrations are widely used to adjust urinary concentrations of chemicals or their metabolites. The formation of urinary creatinine and the ways in which various factors may affect its concentration are important. The rate of creatinine formation is fairly constant, but this rate decreases with age in adults (Alessio et al., 1985). Researchers found a high correlation between urinary creatinine concentrations and muscle mass (Edwards and Whyte, 1959). In addition, men have higher levels of creatinine than women and persons with a high red meat intake have higher levels in comparison with persons with a low red meat intake (Bjornsson, 1979; Lykken et al., 1979). Furthermore, if an analyte is excreted predominantly through passive secretion in the kidney, the analyte secretion will vary with urine flow rate and creatinine adjustment will not correct for urine dilution. Due to these reasons the use of creatinine-adjusted metabolite concentrations in order to accurately compare exposures among the study participants remains a subject of research

(Barr et al., 2005; Boeniger et al., 1993). Within this study, biomarker concentrations were presented both corrected and uncorrected for creatinine levels.

5.4 STATISTICAL ANALYSIS

The data from the questionnaires and the results from the chemical analysis of the urine samples were linked to each other via the identification number and were put together in one central database. When all data was collected, this database was cleaned whereby all data were checked for intolerable values. Afterwards the data were used for statistical analysis. Urinary biomarker data were used as a direct indicator of mycotoxin exposure, without relating it yet to food consumption. These obtained biomarker concentrations were presented both uncorrected and corrected for creatinine levels (the latter to correct for variation in urinary concentration). When two different concentrations were obtained (cf. two LC-MS/MS methods) the highest concentrations were used for further calculations (= worst case). The concentration of DON15GlcA was based on a calibration curve using DON3GlcA, because only a small amount of standard was available.

Additionally, mycotoxin exposure of different subgroups was compared to investigate whether exposure of these subgroups was significantly different using non-parametric tests being the Mann-Whitney U and Kruskal-Wallis tests. The association between BMI and mycotoxin exposure was investigated using a Spearman correlation analysis. Furthermore, this study investigated if the consumption of different food items or food groups could predict the concentrations measured in urine. The population was divided in two classes for each food commodity (consumers and non-consumers). The normality of each class was tested using the Shapiro-Wilkinson test. In a next step, the mycotoxin concentrations (both corrected and uncorrected for creatinine) were compared between the different classes using the non-parametric Mann-Whitney-U test. When less than 5 individuals consumed a specific food commodity, the results of the statistical analysis were not reported. Data was analysed using SPSS software version 21 (p-value < 0.05 was considered as statistically significant). All results were communicated to the authorities.

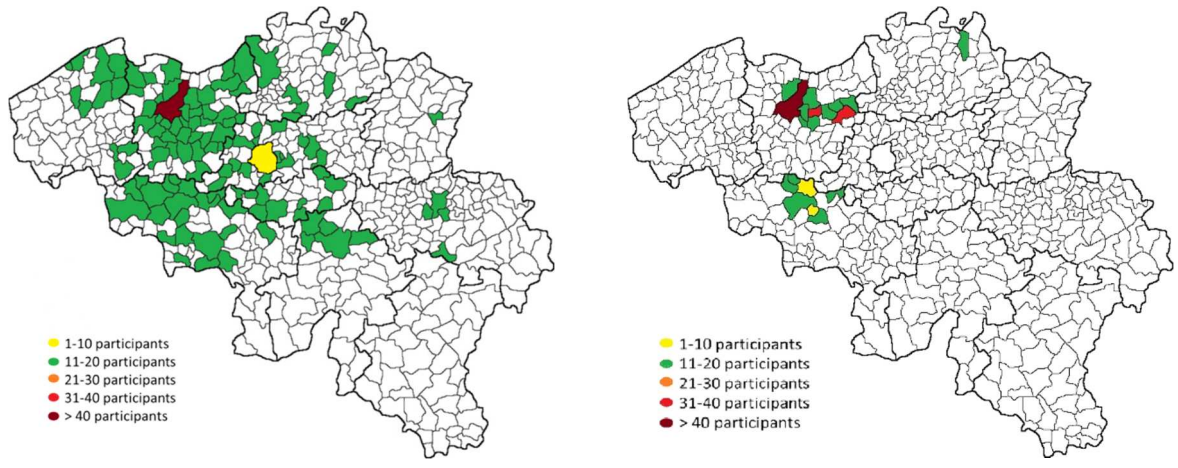
CHAPTER 6

HUMAN BIOMONITORING OF MULTIPLE MYCOTOXINS IN THE BELGIAN POPULATION: RESULTS OF THE BIOMYCO STUDY

6.1 POPULATION CHARACTERISTICS

A sample of 217 children, aged 3-12 years, were enrolled for the study. Based on the exclusion criteria, seven of the recruited children were excluded (age, kidney problem, other participating family member). Another 37 children forgot to collect their urine. Overall 173 subjects returned a urine sample and a completed questionnaire between January 2013 and March 2014. Twelve of these children had no valid urine samples (too diluted, too concentrated, not enough volume) and 6 children did not fill in all the necessary data. A total of 155 children were included (118 from Flanders and 37 from Wallonia) consisting of 67 boys and 88 girls.

Figure 6.1. Location of sampling in Belgium



ADULTS (n=239) CHILDREN (n=155)

Table 6.1. The socio-demographical data of the children and adults at time of sampling are compared to the socio-demographical distribution needed (see table 5.1.)

ADULTS (n = 239)				
Age		**19-34 years**	**35-50 years**	**51-65 years**
	Participated	91 (38 %)	99 (41 %)	49 (21 %)
	Needed	32 %	37 %	31 %
Region		**Flanders**	**Wallonia**	**Brussels**
	Participated	173 (72 %)	50 (21 %)	16 (7 %)
	Needed	60 %	32 %	8 %
Gender		**Male**	**Female**	
	Participated	106 (44 %)	133 (56 %)	
	Needed	50 %	50 %	
CHILDREN (n = 155)				
Age		**3-6 years**	**7-9 years**	**10-12 years**
	Participated	65 (42 %)	46 (30 %)	44 (28 %)
	Needed	40 %	30 %	30 %
Region		**Flanders**	**Wallonia**	**Brussels**
	Participated	118 (76 %)	37 (24 %)	0 (0 %)
	Needed	56 %	35 %	9 %
Gender		**Male**	**Female**	
	Participated	67 (43 %)	88 (57 %)	
	Needed	51 %	49 %	

The second population sample included adults aged 19 to 65 years. A number of 427 adults were enrolled for this study. Five of the recruited adults could not participate according to the exclusion criteria (contact with feed, problems with liver, bile or kidney) and another 131 adults did not show up or forgot to collect their urine. Overall 291 subjects returned a urine sample and a completed questionnaire between January 2013 and March 2014. Fifty two adults had no valid urine samples (too diluted, too concentrated, not enough volume). A total of 239 adults were included (173 in Flanders, 50 in Wallonia and 16 in Brussels) consisting of 106 men and 133 women. The location of sampling in Belgium is presented in figure 6.1. and the flow chart describing the selection procedures is illustrated in figure 6.2. In table 6.1. the socio-demographical data of the children and adults at time of sampling are compared to the socio-demographical distribution needed (table 5.1.). It has to be noted that Brussels accounts for only 8 % of the Belgian population. Flanders has most inhabitants (58 %), whereas Wallonia has less inhabitants (33 %).

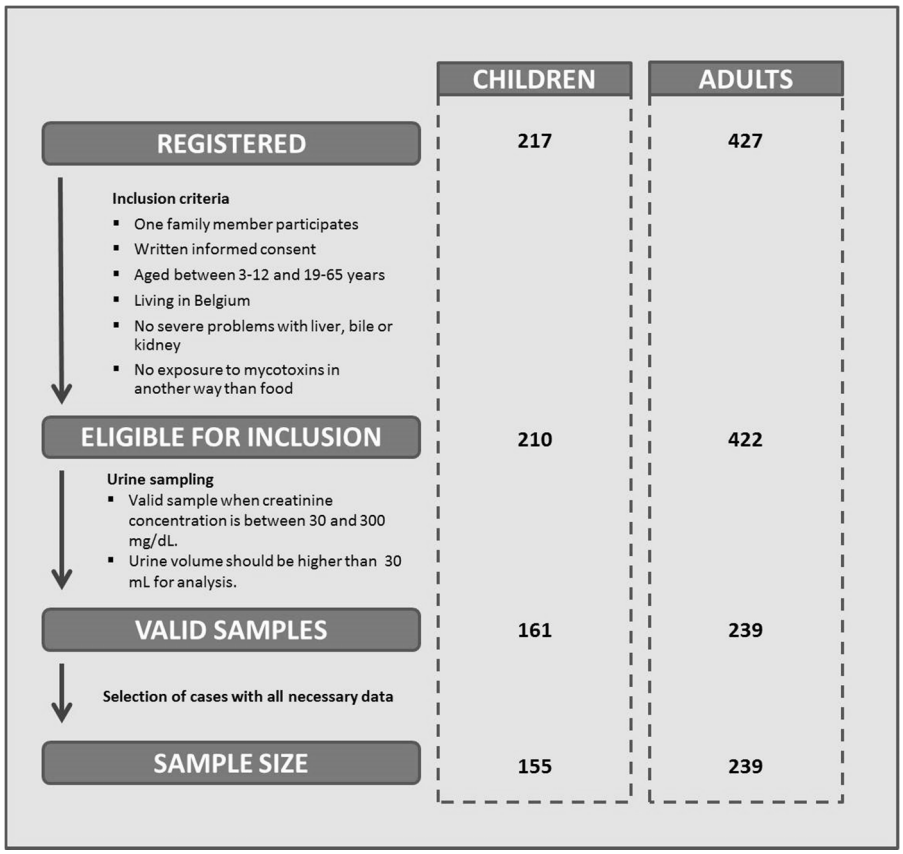


Figure 6.2. Flow chart describing the selection procedure of the studied sample (n=394)

6.2 PREVALENCE OF MYCOTOXINS IN BELGIAN URINE

In total, 394 urine samples from children and adults living in Belgium were analysed for the presence of 33 urinary mycotoxins and their metabolites. Nine out of 33 mycotoxins were detected whereby DON, OTA, CIT and their metabolites DON3GlcA, DON15GlcA, DOMGlcA and HO-CIT were the most frequently detected. DON15GlcA was the main urinary DON biomarker found and for the first time DOMGlcA was detected in urine of children. Furthermore α-ZEL was detected in one urine sample and β-ZEL14GlcA in two samples. AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, FB_1, FB_2, FB_3, HFB_1, DAS, 3ADON, 3ADON15GlcA, 15ADON, 15ADON3GlcA, DOM-1, FusX, HT-2, OTα, T-2, ZEN, ZEN14GlcA, α-ZEL7GlcA, α-ZEL14GlcA and β-ZEL could not be detected or quantified in urine samples from the Belgian population. The incidence, mean, median, minimum and maximum levels of these mycotoxins are summarised in table 6.2 (uncorrected and corrected for creatinine) whereby only data above LOQ were used. The concentration of DON15GlcA was based on a calibration curve using

DON3GlcA, because only a small amount of standard was available. When two different concentrations were found for DON (21/198), OTA (1/163) and α-ZEL (1/1) (cfr. two LC-MS/MS methods), the highest concentrations (= worst case) were used in further calculations. Examples of chromatograms of contaminated urine samples are presented in table 6.3.

An overall incidence of DON was measured in 70 % of the children and 37 % of the adults with a mean concentration of 5.5 ng/mg creatinine for children and 6.1 ng/mg creatinine for adults. DON15GlcA was the most frequently detected biomarker (in 100 % of the samples) with mean levels of 65.3 and 50.1 ng/mg creatinine for children and adults respectively. While DON15GlcA is the main biomarker of DON in humans, DON3GlcA was detected in 91 % of the urine samples from children with a mean concentration of 12.3 ng/mg creatinine. For adults, 77 % of the urine samples were positive for DON3GlcA with a mean concentration of 6.7 ng/mg creatinine. For the first time DOMGlcA was detected in urine samples of children. The incidence in children was 17 % with a mean concentration of 100.9 ng/mg creatinine. For adults, DOMGlcA was detected in 22 % of the samples with a mean concentration of 25.0 ng/mg creatinine.

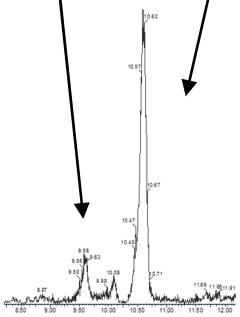
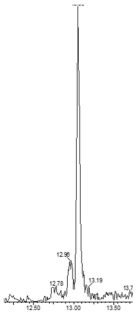
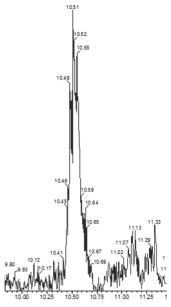
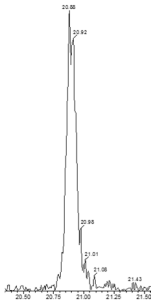
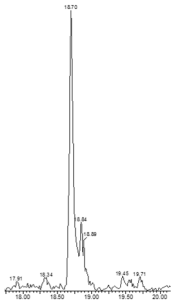
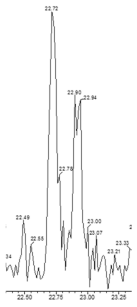
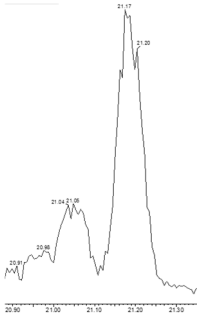
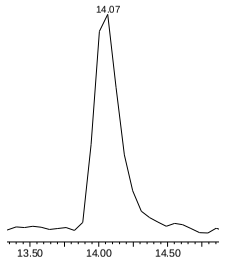
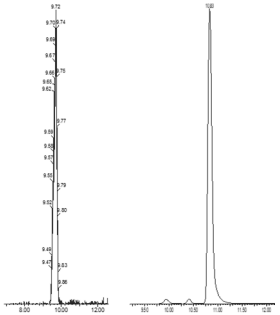
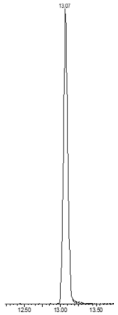
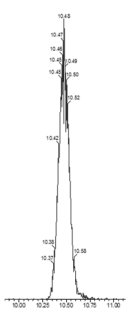
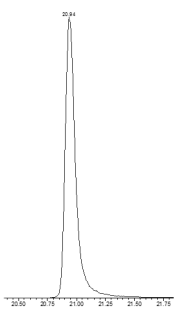
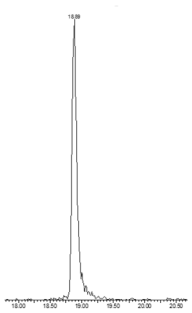
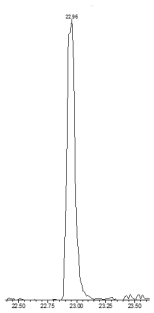
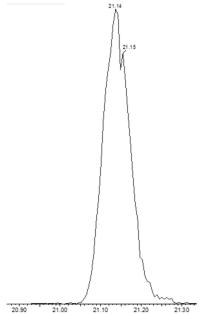
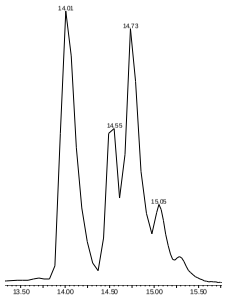
Results showed that 51 % of the urine samples from children and 35 % of the urine samples from adults were OTA contaminated. The mean concentration was 83.2 pg/mg creatinine and 36.4 pg/mg creatinine for children and adults respectively. Despite its high occurrence (72 % for children and 59 % for adults), the average urinary concentrations of CIT were low (same range as OTA), being 39.8 pg/mg creatinine and 73.3 pg/mg creatinine for children and adults respectively. Also the metabolite of CIT, namely HO-CIT, was detected in 6 % of the children and 12 % of the adults. The mean concentration of HO-CIT was tenfold higher than CIT. α-ZEL was only found in one sample and β-ZEL14GlcA in two urine samples of adults. None of these mycotoxins were found in children.

Table 6.2. Mycotoxin contamination incidence and levels in urine of the Belgian population uncorrected and corrected for creatinine levels

Population group	Parameter	CIT	HO-CIT*	DON	DON3GlcA	DON15GlcA	DOMGlcA	OTA	α-ZEL	β-ZEL14GlcA
Children	**Incidence**	112/155	7/124	109/155	141/155	155/155	26/155	79/155	n.d.	n.d.
	% samples >LOQ	72	6	70	91	100	17	51	n.d.	n.d.
	Uncorrected for creatinine	pg/mL	pg/mL	ng/mL	ng/mL	ng/mL	ng/mL	pg/mL	ng/mL	ng/mL
	Mean	31.4	550.7	5.2	10.6	58.4	91.7	79.5	n.d.	n.d.
	Median	21.2	494.3	3.9	7.8	42.6	24.0	29.5	n.d.	n.d.
	Minimum	1.6	259.4	0.5	0.7	4.3	1.1	3.8	n.d.	n.d.
	Maximum	392.8	887.3	32.5	43.0	343.0	526.1	3683.0	n.d.	n.d.
	Corrected for creatinine	pg/mg	pg/mg	ng/mg	ng/mg	ng/mg	ng/mg	pg/mg	ng/mg	ng/mg
	Mean	39.8	810.2	5.5	12.3	65.3	100.9	83.2	n.d.	n.d.
	Median	21.7	439.9	4.1	9.7	51.7	28.2	28.1	n.d.	n.d.
	Minimum	2.0	268.8	0.6	0.7	4.6	1.4	4.0	n.d.	n.d.
	Maximum	415.7	2029.0	27.4	53.0	301.7	559.1	3828.1	n.d.	n.d.
Adults	**Incidence**	140/239	14/120	89/239	184/239	238/239	53/239	84/239	1/239	2/239
	% samples >LOQ	59	12	37	77	100	22	35	0.4	0.8
	Uncorrected for creatinine	pg/mL	pg/mL	ng/mL	ng/mL	ng/mL	ng/mL	pg/mL	ng/mL	ng/mL
	Mean	56.7	752.0	3.9	7.5	53.8	16.9	27.8	5.0	0.8
	Median	17.6	560.3	1.7	4.4	31.2	5.8	15.2	5.0	0.8
	Minimum	2.2	143.1	0.5	0.5	1.1	0.6	2.7	5.0	0.6
	Maximum	1398.0	2117.7	129.8	126.2	460.8	172.0	368.1	5.0	1.0
	Corrected for creatinine	pg/mg	pg/mg	ng/mg	ng/mg	ng/mg	ng/mg	pg/mg	ng/mg	ng/mg
	Mean	73.7	739.0	6.1	6.7	50.1	25.0	36.4	4.3	0.9
	Median	19.9	530.5	2.1	3.9	29.8	4.3	15.5	4.3	0.9
	Minimum	1.6	92.9	0.2	0.3	1.3	0.5	1.8	4.3	0.7
	Maximum	1494.3	2465.6	327.0	88.9	546.2	458.4	927.4	4.3	1.1

*n.d. not detected; LOQ limit of quantification; All the presented concentrations were only those above LOQ (CIT-OTA: 3 pg/mL; HO-CIT: 30 pg/mL; DON-DON3GlcA-DON15GlcA-DOMGlcA- β-ZEL14GlcA: 500 pg/mL; α-ZEL: 220 pg/mL); *not all samples were analysed for HO-CIT*

Table 6.3. Chromatograms showing peaks of naturally contaminated urine samples and standard solutions (20 ng/mL) of DON3GlcA, DON15GlcA, DON, DOMGlcA, CIT, HO-CIT, OTA, β-ZEL14GlcA and chromatograms of a naturally contaminated and spiked urine sample (20 ng/mL) of α-ZEL

Mycotoxin	DON3GlcA - DON15GlcA	DOMGlcA	DON	CIT	HO-CIT	OTA	β-ZEL14GlcA	α-ZEL
Naturally contaminated urine sample								
Standard solution or spiked urine sample (20 ng/mL)								

6.3 PATTERNS IN MYCOTOXIN EXPOSURE

Table 6.4. shows moderate to strong correlations (r= 0.5-0.8) between urinary concentrations of DON, DON3GlcA and DON15GlcA. These correlations were both found in adults and children, whereby the urinary concentrations were corrected and uncorrected for creatinine. These correlations suggest that humans are efficiently conjugating DON with glucuronic acid and confirm earlier reports (Huybrechts et al., 2014).

Table 6.4. Pairwise comparison of the different biomarkers using the p-value(s)

Population group	Mycotoxin	Uncorrected for creatinine			Corrected for creatinine		
		n	r	p	n	r	p
Children	**DON-DON3GlcA**	155	0.572	< 0.001	106	0.566	< 0.001
	DON-DON15GlcA	155	0.671	< 0.001	109	0.607	< 0.001
	DON3GlcA-DON15GlcA	155	0.751	< 0.001	141	0.718	< 0.001
Adults	**DON-DON3GlcA**	239	0.433	< 0.001	76	0.309	0.007
	DON-DON15GlcA	239	0.410	< 0.001	89	0.226	0.033
	DON3GlcA-DON15GlcA	239	0.717	< 0.001	184	0.795	< 0.001

n: amount of urine samples; r: Spearman rank correlation coefficient; p < 0.05 is statistical significant; Half the value of their respective LOD's (=100 pg/mL) were assigned to samples with no quantifiable biomarkers. When concentrations corrected for creatinine were used, only positive samples were used (>LOQ).

6.4 RISK ASSESSMENT

A risk assessment was performed by comparing the estimated dietary intake of DON and OTA with their TDI. Exceeding the TDI can imply a possible health risk for the Belgian population. The dietary intake of DON and OTA was estimated using the urinary concentrations. The calculated intake was compared to the following TDI's:

- DON (including acetylated DON's) = 1 µg/kg BW/day (FAO/WHO, 2010)
- OTA = 0.017 µg/kg BW/day (Schlatter et al., 1996), based on TWI of 0.12 µg/kg BW/week (EFSA, 2006)

Currently, there are different methods available to calculate the estimated exposure through urinary levels. Following calculations were reported:

(1) Estimated dietary intake DON (µg/kg. BW/day) $= \frac{DON * V}{ER * BW * 1000}$

Reported by Gratz et al. (2014); Wallin et al. (2013); Rodriguez-Carrasco et al. (2014b); Turner et al. (2010b)

(2) Estimated dietary intake DON (µg/kg. BW/day) $= \frac{(DON + DON3GlcA + DON15GlcA + DOMGlcA) * V}{ER * BW * 1000}$

Reported by Ezekiel et al. (2014)

(3) Estimated dietary intake DON (µg/kg. BW/day) $= \frac{(DON + ((DON3GlcA + DON15GlcA) * 0.63) + (DOMGlcA * 0.65)) * V}{ER * BW * 1000}$

Reported by Warth et al. (2012b)

(4) Estimated dietary intake DON (µg/kg. BW/day) $= \frac{(DON + ((DON3GlcA + DON15GlcA) * 0.63) + (DOMGlcA * 0.65)) * V}{ER * BW * CF * 1000}$

Reported by Sarkanj et al. (2013)

(5) Estimated dietary intake OTA (µg/kg. BW/day) $= \frac{OTA * V}{ER * BW * 1.000.000}$

Reported by Ezekiel et al. (2014)

Assumptions made:

ER = % of ingested DON excreted in urine = 72 % (Turner et al., 2010b)

= % of ingested OTA excreted in urine = 50 % (Schlatter et al., 1996)

V = daily urine production of adults is 1500 mL and children is 1000 mL (Turner et al., 2010b)

BW = body weight (kg) reported in questionnaire

CF = concentration factor, morning urine is 2 times more concentrated than urine during day (Warth et al., 2013a)

DON/DON3GlcA/DON15GlcA/DOMGlcA = urinary concentration not corrected for creatinine (ng/ml)

OTA = urinary concentration not corrected for creatinine (pg/ml)

Factor 0.63 and 0.65 was obtained by calculating DON-equivalents (see section 7.1)

It has to be highlighted that these calculations are based on excretion rate assumptions and that inter-individual variations were not taken into account. Due to the lack of toxicokinetic data on mycotoxins in human, there are still a lot of uncertainties that should be taken into account when perform a risk assessment based on urinary mycotoxin levels. The dietary intake of DON was in formula 1 estimated based on the individual urinary DON levels. The use of this formula leads to an underestimation of the DON exposure as no metabolites were taken into account. Formula 2 does not take into account the molar masses of the DON metabolites. Furthermore in formula 1, 2, 3 and 5 the assumption was made that morning urine is equally concentrated than urine collected during the day, leading to an overestimation. In formula 4 the difference in concentration of morning urine and urine collected during the day was taken into account. Additionally, the presence of unknown metabolites can lead to an underestimation. The quantification of DON15GlcA was based on the concentration of DON3GlcA, because of the small amount of standard solution available. When enough standard solution of DON15GlcA is available in the future, these data can be corrected. Because no standard method is available at this moment for calculating the estimated exposure through urinary concentrations, the dietary exposure within the BIOMYCO study was calculated in two different ways (formulae 3 and 4). This approach will enable the comparison with other exposure assessments performed in different European countries. All the different estimated intakes are presented in table 6.5.

The estimated intake for DON varies between 0.11 – 19.57 µg/kg BW/day for children and 0.03 – 10.08 µg/kg BW/day for adults. Of total exposed subjects 56-69 % of children and 16-39 % of the adults were estimated to exceed the TDI. For OTA the individually urinary level was used to carry out an exposure assessment. The estimated intake for OTA was between 0.2 – 100 ng/kg BW/day for children and 0.1 – 21 ng/kg BW/day for adults. In total 1 % of the Belgian population exceeded the TDI for OTA.

Due to the lack of toxicokinetic data for CIT, no risk assessment could be performed for this mycotoxin.

In general, in this kind of exposure assessments young children need special attention because of the quantitative higher food intake per kg body weight. In order to perform more accurate estimations, more research needs to be done in order to collect more information about the human metabolism of mycotoxins, especially for CIT. More exposure assessment studies are needed in order to compare the different estimates and to evaluate the variability amongst people.

Table 6.5. Estimated dietary intake of DON and OTA based on urinary levels. The intake was compared to the TDI.

Estimated dietary intake BIOMYCO		Formula	% exceeding TDI	References with similar calculation method
DON (µg/kg BW/day)	Children = 0.17 – 19.57 (mean = 3.26)	(3)	69 % (100/144)	Warth et al. (2012a)
	Adults = 0.06 – 10.08 (mean = 1.24)		29 % (68/236)	
	Children = 0.11 – 12.73 (mean = 1.63)	(4)	56 % (81/144)	Sarkanj et al. (2013)
	Adults = 0.03 – 5.04 (mean = 0.62)		16 % (39/236)	
OTA (ng/kg BW/day)	Children = 0.2 – 100 (mean = 5.41)	(5)	1 % (1/71)	Ezekiel et al. (2014), Solfrizzo et al. (2014)
	Adults = 0.1 – 21 (mean = 1.27)		1 % (1/81)	

CHAPTER 7

URINARY MYCOTOXIN BIOMARKERS IN RELATION TO FOOD CONSUMPTION AND SOCIO-DEMOGRAPHICAL CHARACTERISTICS IN BELGIAN CHILDREN AND ADULTS

7.1 URINARY BIOMARKERS IN RELATION TO SOCIO-DEMOGRAPHICAL CHARACTERISTICS

Socio-demographical data of the children and adults at time of sampling are summarized in table 7.1. The incidence, mean, median, minimum and maximum levels of the urinary mycotoxin biomarkers are presented in table 6.2. (uncorrected and corrected for creatinine). Because metabolites are formed in the human body and are not present in food, DON-equivalents were calculated using the concentrations of DON3GlcA, DON15GlcA and DOMGlcA. These equivalents were further used to investigate the relationship between socio-demographical characteristics and urinary biomarkers. The same calculations were performed for CIT.

$$DON - equivalents$$

$$(6) = DON + \frac{(DON3GlcA + DON15GlcA) * MW\ DON}{MW\ DONGlcA} + \frac{(DOMGlcA) * MW\ DON}{MW\ DOMGlcA}$$

$$CIT - equivalents$$

$$(7) = CIT + \frac{(HO-CIT) * MW\ CIT}{MW\ HO-CIT}$$

With

DON/DON3GlcA/DON15GlcA/DOMGlcA = urinary concentration uncorrected for creatinine (ng/mL)

CIT/HO-CIT = urinary concentration uncorrected for creatinine (pg/mL)

MW = molecular weight (DON = 296, DONGlcA = 472, DOMGlcA = 456, CIT = 250, HO-CIT = 266)

Table 7.1. Socio-demographical data of the children and adults at time of sampling

CHILDREN (n=155)		ADULTS (n=239)	
Gender		**Gender**	
Boy	*67*	Male	*106*
Girl	*88*	Female	*133*
Age		**Age**	
3-6 years	*65*	*19-34 years*	*91*
7-9 years	*46*	*35-50 years*	*99*
10-12 years	*44*	*51-65 years*	*49*
Region		**Region**	
Flanders	*118*	*Flanders*	*173*
Wallonia	*37*	*Wallonia*	*50*
Brussels	*0*	*Brussels*	*16*
Mean weight in kg (min-max)	27 (12-55)	**Mean weight in kg (min-max)**	72 (40-129)
Mean height in cm (min-max)	127 (93-158)	**Mean height in cm (min-max)**	173 (149-205)
Mean BMI kg/m² (min-max)	16 (12-25)	**Mean BMI kg/m² (min-max)**	24 (17-42)
Mean BMI z-score (min-max)	0.837 (0.003-3.385)		
Dietary restrictions		**Dietary restrictions**	
Gluten free	*1*	*Gluten free*	*3*
Lactose free	*0*	*Lactose free*	*5*
Egg white free	*2*	*Egg white free*	*1*
Losing weight	*0*	*Losing weight*	*5*
Low-fat or cholesterol	*1*	*Low-fat or cholesterol*	*4*
Low-salt	*0*	*Low-salt*	*2*
Diabetes	*0*	*Diabetes*	*0*
No nuts	*2*	*No sulfites*	*1*
No fish	*1*	*No fruit*	*1*
No information	*8*	*No caffeine*	*1*
		No information	*10*
Diets		**Diets**	
West-European	*107*	*West-European*	*135*
Mediterranean	*2*	*Mediterranean*	*2*
Oriental	*0*	*Oriental*	*0*
Halal	*2*	*Halal*	*0*
Kosher	*0*	*Kosher*	*0*
Limited meat intake	*2*	*Limited meat intake*	*13*
Vegetarian	*2*	*Vegetarian*	*10*
Vegan	*0*	*Vegan*	*1*
Combination	*30*	*African*	*1*
No information	*10*	*Combination*	*68*
		No information	*9*
		Smoking behaviour	
		Smokers	*19*
		Non-smokers	*215*
		No information	*5*
		Pregnant women	5
		Breast-feeding women	1

The urinary biomarker concentrations measured in this Belgian population differed significantly among age and gender. Children from 3 to 6 years old were exposed to lower levels of DON (based on DON-equivalents, p-value 0.032) in comparison with children from 10 to 12 years old. No significant differences concerning urinary biomarker levels between girls and boys were found. In contrast, in the adult group men showed significantly higher levels of DON (based on DON-equivalents, p-value 0.014) in comparison with women. Adults between 19 and 34 years old had significantly lower urinary levels of OTA (p-value 0.012) than adults from 51 until 65 years old. All concentrations used for these data analysis were uncorrected for creatinine due to the fact that creatinine was correlated to some of these socio-demographical characteristics. Additionally a significantly negative correlation was found between BMI and creatinine-adjusted levels of DON3GlcA ($r= -0.167$, $p= 0.025$, $n= 181$) and DON15GlcA ($r= -0.156$, $p= 0.021$, $n= 235$) in adults and creatinine-adjusted levels of CIT ($r= -0.219$, $p= 0.030$, $n= 99$) in children using a Spearman correlation analysis. A positive correlation was found between BMI and creatinine-adjusted HO-CIT levels ($r= 0.571$, $p= 0.041$, $n= 13$) in adults.

Furthermore, the difference in exposure between different diets was investigated. Most of the Belgian people consumed a West-European diet (table 7.1.). Also Mediterranean, Halal, African, limited meat intake, vegetarian, vegan diets or a combination were reported in the questionnaire. Because of the limited number of subjects, a statistical analysis could not be performed for some of these subgroups. For children only a significant difference could be seen between children who followed a West-European diet (n=87) and children who followed a combination of different diets (n=21), whereby children with only a West-European diet had significantly higher levels of creatinine-adjusted DON in their urine (p=0.034). However, it has to be remarked that this trend should be confirmed by future research. The difference in exposure between different dietary restrictions for children and adults and between breast-feeding women and no breast-feeding women could not be analysed due to the limited number of subjects within these subgroups. A significant difference could be seen for pregnant women whereby the levels of CIT-equivalents ($p= 0.013$, $n=5$) were lower in comparison with non-pregnant women. Also the urinary levels of creatinine-adjusted OTA were lower for the pregnant women ($p=0.045$, $n=3$). However, it

has to be remarked that the number of subjects was too small to have an accurate analysis. This trend should be confirmed by future research. Finally, no significant difference in exposure between smokers and non-smokers could be seen.

7.2 URINARY MYCOTOXIN BIOMARKERS IN RELATION TO FOOD CONSUMPTION

The total intake (g/day or mL/day) of the 43 food commodities reported by Belgian adults and children (n=394) was calculated based on the data collected with the FFQ (table 7.2). The incidence and mean levels of the urinary mycotoxin biomarkers are presented in table 6.2 (uncorrected and corrected for creatinine). Because metabolites are formed in the human body and are not present in food, DON- and CIT-equivalents were used to investigate the relationship between food intake and urinary biomarkers (see 7.1. for formulae).

The urinary OTA levels were compared between consumers and non-consumers of 43 different food commodities consumed in the month previously to the urine collection. All significant differences found, are presented in table 7.3. Children who consumed raisin bread, rusk or whole grain rice showed higher OTA levels in comparison with non-consumers. Adults who consumed pancakes, had higher levels of urinary OTA. Despite the reported occurrence of OTA in dried fruits and nuts, children who consumed assorted nuts with raisins had lower levels of OTA than the non-consumers. Children who consumed white bread and vegetarian burgers had lower levels of OTA in comparison with the non-consumers. The same trend was seen by the adults namely consumers of cereal based products (white bread, wholemeal bread, pizza or durum and white pasta) had significantly lower levels of OTA.

Table 7.2. Total intake (g/day or mL/day) of 43 food commodities reported by Belgian adults and children (n=394) using a food frequency questionnaire

Food group	ADULTS (n=239)										CHILDREN (n=155)									
	Total intake (g/day or mL/day) previous month					Total intake (g/day or mL/day) previous 24h					Total intake (g/day or mL/day) previous month					Total intake (g/day or mL/day) previous 24h				
	Consumers	Mean	Median	Min	Max	Consumers	Mean	Median	Min	Max	Consumers	Mean	Median	Min	Max	Consumers	Mean	Median	Min	Max
Coffee	171	315	250	4	1250	155	366	375	63	1250	6	57	36	9	125	4	125	125	125	125
Soy drinks	45	77	36	4	375	23	253	250	63	1250	27	102	36	9	500	13	365	250	125	1000
Beer	142	115	50	9	1037	29	470	330	125	1320	0	0	0	0	0	0	0	0	0	0
Wine	190	63	36	4	625	66	230	250	63	1000	0	0	0	0	0	0	0	0	0	0
Muesli and cruesli	77	17	9	1	157	27	45	40	20	80	14	7	3	1	17	6	113	40	20	360
Cornflakes	64	14	6	1	90	23	44	30	15	90	101	11	4	1	60	30	41	30	15	150
White bread	188	46	19	1	424	94	105	80	20	445	117	73	42	1	410	51	95	80	20	297
Rye bread	50	18	9	2	78	9	73	66	33	132	12	17	5	2	52	2	132	132	33	231
Wholemeal bread	197	109	104	2	429	147	135	132	33	429	97	80	66	2	509	53	90	80	33	231
Raisin bread	68	9	7	2	28	9	84	66	33	132	33	7	5	2	19	8	95	116	33	132
Soft white bread	105	11	9	2	69	15	89	80	20	226	105	19	11	2	146	37	96	80	20	400
Viennoiseries	161	14	9	2	111	29	95	65	33	195	100	14	9	5	223	27	106	65	65	390
Rusk	68	6	2	1	43	18	29	30	10	50	43	5	2	1	40	6	30	15	10	70
Cereal bar	26	6	4	2	21	5	60	25	25	150	10	6	5	2	11	3	67	25	25	150
Ginger bread	62	6	3	1	46	7	38	46	12	69	27	111	92	46	276	2	69	69	23	115
Biscuits	185	12	9	1	60	87	28	20	10	70	118	16	16	1	70	63	20	20	10	40
Pancake	95	14	13	2	51	7	116	120	30	240	95	11	9	2	51	17	134	120	60	420
Cake	129	6	4	1	51	23	40	30	15	90	101	7	4	1	30	18	43	30	15	120
Pastry and pie	169	16	8	2	197	27	142	115	29	460	68	12	8	8	49	15	130	115	115	230
Whole grain rice	63	16	11	2	116	6	150	165	90	210	28	16	9	2	129	3	200	210	90	300
White rice	166	20	13	2	300	17	146	120	90	300	105	12	9	2	77	12	108	105	60	180
Wholemeal pasta	99	55	43	7	386	10	330	300	150	900	45	25	21	5	64	22	181	150	75	900
White pasta	190	78	43	2	386	49	271	300	50	900	120	57	38	5	386	22	297	150	75	2100
Polenta	13	8	6	2	17	1	30	30	30	30	4	9	8	2	17	1	90	90	90	90
Pizza and durum	160	25	21	1	214	11	150	120	38	360	95	15	11	3	154	10	243	140	30	750
Tortilla	42	7	6	1	34	6	60	60	40	80	17	4	3	1	6	2	100	100	40	160
Soy dessert	36	37	18	2	250	6	208	125	125	500	21	47	18	9	196	5	300	125	125	875
Tofu	31	10	9	3	64	5	64	80	20	120	9	13	11	5	22	2	303	303	80	525
Tempeh	5	10	11	5	17	0	0	0	0	0	1	11	11	11	11	3	140	120	20	280
Vegetarian burger	38	11	5	5	59	4	75	75	75	75	13	9	5	5	32	0	0	0	0	0
Assorted nuts with raisins	67	8	4	1	47	8	38	20	20	100	15	4	3	1	9	2	30	30	20	40
Peanuts and peanut cheese	91	6	4	1	34	20	47	40	20	80	46	3	3	1	31	7	43	20	20	120
Pistachio nuts	51	2	1	1	6	3	17	20	10	20	18	2	1	1	4	3	50	40	10	100
Other nuts	117	11	4	2	94	30	47	30	30	210	52	5	4	2	39	4	45	30	30	90
Raisins	80	3	2	1	48	6	20	12	12	48	40	4	3	1	24	9	30	24	12	96
Dried fruit	62	8	2	1	79	15	40	32	8	120	15	4	1	1	34	2	24	24	8	40
Popcorn	20	4	4	1	9	0	0	0	0	0	25	3	2	1	7	4	45	50	10	70
Corn-based crisps	98	5	3	1	39	13	31	30	15	60	42	3	2	1	9	4	23	23	15	30
Seitan	19	3	2	1	16	2	23	23	15	30	8	4	2	1	25	37	152	175	15	175
Quorn	38	10	6	1	51	6	80	80	80	80	16	18	9	1	100	2	77	80	20	80
Maize	62	13	5	3	107	2	53	40	20	100	24	11	5	1	32	7	60	60	40	80
Paprika powder	22		No information			130	4	2	2	12	15		No information			61	4	2	2	22
Chilli powder	14		No information			68	3	2	2	12	1		No information			15	2	2	2	4

The urinary levels of DON, CIT, DON-equivalents and CIT-equivalents between consumers and non-consumers of 43 different food commodities consumed on the day prior to the urine collection were compared, for children and adults. All significant differences found are presented in table 7.4. Children who consumed raisin bread had higher levels of DON-equivalents in their urine and higher levels of urinary DON were present in the urine of children who consumed soft white bread. On the contrary, children who consumed muesli, cruesli and cornflakes had lower levels of DON and consumers of raisins had lower levels of DON-equivalents. For the adults, no significant differences on the level of food consumption could be found for DON-equivalents. Consumers of beer, corn-based crisps and chilli powder had significantly higher levels of DON. This is in contrast with adults who drank coffee, they had significantly lower levels.

Within the BIOMYCO study significantly higher CIT levels were present in the urine of children consuming biscuits, whereas consumers of pizza or durum and seitan had significantly lower levels of CIT. The consumption of cornflakes and white pasta showed significantly higher levels of CIT-equivalents in urine of children in comparison with non-consumers. The consumers of viennoiseries and corn-based crisps had significantly lower levels of respectively CIT and CIT-equivalents in adults.

Table 7.3. Pairwise comparison of urinary OTA levels between consumers and non-consumers of different food commodities

(consumption in the month prior to the urine collection)

Population	Food commodity		Not corrected for creatinine	Corrected for creatinine
			p	p
Children	White bread	Consumers (n=117) < non-consumers (n=38)	0.013	
	Raisin bread	Consumers (n= 33) > non-consumers (n=122)	0.011	
	Rusk	Consumers (n= 43) > non-consumers (n=112)	0.035	
	Whole grain rice	Consumers (n= 105) > non-consumers (n=50)	0.037	
	Vegetarian burger	Consumers (n= 9) < non-consumers (n=70)		0.031
	Assorted nuts with raisins	Consumers (n= 7) < non-consumers (n=72)		0.012
Adults	White bread	Consumers (n= 188) < non-consumers (n=51)	0.007	
	Wholemeal bread	Consumers (n= 68) < non-consumers (n=16)		0.008
	Pancake	Consumers (n= 95) > non-consumers (n=144)	0.049	
	White pasta	Consumers (n= 190) < non-consumers (n=49)	0.016	
	Pizza or durum	Consumers (n= 64) < non-consumers (n=20)		0.031

Mann-Whitney-U test (based on mean ranks); n: amount of consumers; Half the value of their respective LOD's (=0.5 pg/mL) were assigned to samples with no quantifiable biomarkers. When concentrations corrected for creatinine were used, only positive samples were used (>LOQ), p-value < 0.05 was considered as statistically significant.

Table 7.4. Pairwise comparison of urinary DON and CIT levels between consumers and non-consumers of different food commodities (consumption in the day prior to the urine collection)

Population	Mycotoxin	Food commodity		Not corrected for creatinine	Corrected for creatinine
				p	p
Children	DON	Muesli and cruesli	Consumers (n=5) < non-consumers (n=104)		0.044
		Cornflakes	Consumers (n=24) < non-consumers (n=85)		0.032
		Soft white bread	Consumers (n=37) > non-consumers (n=118)	0.009	
	DON-equivalents	Raisin bread	Consumers (n=8) > non-consumers (n=147)		0.012
		Raisins	Consumers (n=9) < non-consumers (n=146)	0.032	0.032
	CIT	Biscuits	Consumers (n=44) > non-consumers (n=68)		0.012
		Pizza or durum	Consumers (n=9) < non-consumers (n=103)		0.017
		Seitan	Consumers (n=37) < non-consumers (n=118)	0.002	
	CIT-equivalents	Cornflakes	Consumers (n=27) > non-consumers (n=97)	0.047	
		White pasta	Consumers (n=21) > non-consumers (n=103)	0.012	
Adults	DON	Coffee	Consumers (n=52) < non-consumers (n=37)	0.026	0.003
		Beer	Consumers (n=10) > non-consumers (n=79)		0.013
		Corn-based crisps	Consumers (n=13) > non-consumers (n=226)	0.015	
		Chilli powder	Consumers (n=14) > non-consumers (n=77)	0.043	
	CIT	Viennoiseries	Consumers (n=18) < non-consumers (n=122)		0.046
	CIT-equivalents	Corn-based crisps	Consumers (n=9) < non-consumers (n=135)		0.049

Mann-Whitney-U test (based on mean ranks); n: amount of consumers; Half the value of their respective LOD's (DON=100 pg/mL and CIT = 0.5 pg/mL) were assigned to samples with no quantifiable biomarkers. When concentrations corrected for creatinine were used, only positive samples were used (>LOQ), p-value < 0.05 was considered as statistically significant

PART3

Discussion and future perspectives

CHAPTER 8

GENERAL DISCUSSION

8.1 MAIN FINDINGS AND COMPARISON WITH LITERATURE

8.1.1 Study design

Mycotoxins form a worldwide problem and therefore human exposure to these toxins should be assessed. Human exposure to mycotoxins can be assessed by two major approaches: based on calculations combining mycotoxin contamination data in food with population data on food consumption (De Boevre et al., 2013; Gauchi and Leblanc, 2002; Kuiper-Goodman et al., 2010) and mycotoxin analysis (biomarkers) in biological fluids (e.g. urine).

Within the BIOMYCO study 394 urine samples were collected from healthy children and adults in Belgium for mycotoxin analysis. Participants were asked for their medical history, smoking behaviour, recent medication, dietary intake, diet restrictions and socio-demographical data through a general questionnaire. Furthermore, every participant completed a FFQ to assess the consumption of relevant foods (n=43) on both the day and the month prior to the urine collection.

The biomarker approach is particularly useful as it covers mycotoxin exposure by all routes (oral, dermal, inhalation). Moreover, collection of urine is an easy and non-invasive sampling method. Thanks to the high sensitivity of the recently developed analytical instruments, different LC-MS/MS methods for the detection and quantification of biomarkers in urine were developed in order to assess mycotoxin exposure (Ediage et al., 2012; Gambacorta et

al., 2013; Solfrizzo et al., 2011b; Solfrizzo et al., 2013; Song et al., 2013; Warth et al., 2011; Warth et al., 2013b). These LC-MS/MS methods were validated in pilot surveys on a small-scale. Only recently (past 10 years), studies were increasingly reported whereby the exposure of mycotoxins in different populations was assessed through biomarkers of exposure.

Pena et al. (2006) and Duarte et al. (2012) estimated the exposure of OTA in the Portuguese population, whereas Gilbert et al. (2001) assessed the dietary exposure of OTA in 50 individuals in the UK using a duplicate diet approach. FB_1 was used as an urinary biomarker of exposure in a maize intervention study among South African subsistence farmers (Van der Westhuizen et al., 2011b), whereas sphingoid base levels in humans consuming fumonisin-contaminated maize were investigated in rural areas of the former Transkei in South Africa (Van der Westhuizen et al., 2008). In 2012 exposure assessment to *Fusarium* toxins was performed in 110 women from Iran (Turner et al., 2012). In France, urinary DON and DOM-1 were determined in urine of male farmers (Turner et al., 2010a). Turner et al. (2008b) linked urinary DON with cereal intake in individuals from the UK. Whereas most of these studies focus on the exposure of one mycotoxin, Ediage et al. (2013) conducted a study to investigate the mycotoxin exposure in 220 children in Cameroon whereby 18 analytes were determined in urine samples. Rubert et al. (2011) analysed OTA, DON, T-2, HT-2, ZEN, fumonisins and aflatoxins in urine of 27 volunteers.

In contrast with most of these previously performed studies, the BIOMYCO study is the first study whereby a multi-toxin approach is applied for mycotoxin exposure assessment in adults and children on a large-scale. Moreover, it is the first study that described the exposure to an elaborated set of mycotoxins in the Belgian population. Additionally, studies using biomarkers to assess mycotoxin exposure in European children are – to the authors' knowledge – not yet available. Finally, the BIOMYCO study described for the first time the used study design within a biomarker study in detail.

8.1.2 Prevalence of mycotoxins in Belgian urine

In total, 394 urine samples from children and adults living in Belgium were analysed for the presence of 33 urinary mycotoxins and their metabolites. Nine out of 33 biomarkers were detected whereby DON(-glucuronides), DOMGlcA, OTA, CIT and HO-CIT were the most frequently detected. DON15GlcA was the main urinary metabolite found in 100 % of the samples and for the first time DOMGlcA was detected in urine of children. Furthermore a strong correlation was found between DON and its glucuronides.

Recently, an increasingly number of studies became available whereby the exposure to mycotoxins in different European populations was assessed through urinary biomarkers (see section 3.3). Turner et al. (2008a) performed a pilot survey in the UK whereby 25 healthy adults participated. Urine samples were analysed for **DON** following β-glucuronidase treatment (LOQ 0.6 ng/mL). During consumption of the normal diet, all individuals had detectable urinary levels of DON, while fewer samples were positive (36 %) during the intervention. The geometric mean level during normal diet was 7.2 ng/mg creatinine. Additionally, Turner et al. (2010a) performed a pilot survey in male French farmers (n= 76) whereby DON was detected in 75/76 urine samples (0.5-28.8 ng/mL) and **DOM-1** in 26/76 samples (0.2-2.8 ng/mL). Warth et al. (2011) developed a LC-MS/MS method allowing quantification of both DON (LOD-LOQ 6-20 ng/mL) and **DON3GlcA** (LOD-LOQ 3-10 ng/mL) by a simple **dilute and shoot** approach. The applicability of the method was demonstrated through a pilot survey in Austrian adults whereby the average concentration of total DON (22 % free + 96 % glucuronides) was estimated to be 20.4 µg/L (max. 63 µg/L). **DON15GlcA** (LOD-LOQ 3.2-10.6 ng/mL) could be identified as a major DON metabolite in human urine based on the analysis of these samples. The results of the BIOMYCO project confirmed that glucuronidation of DON at the 15-position is the major detoxification route of DON in adults and children as earlier suggested by Warth et al. (2012a) and Huybrechts et al. (2014). Although we could not find DOM-1 in the Belgian urine samples, DOMGlcA was found in 22 % of the adults. This incidence is comparable with the 25 % found in the pilot survey performed in Belgium (Huybrechts et al., 2014). Our results confirm the suggestion that

DOM-1 derives from DON metabolism by intestinal microbiota, whereby not every person possesses this activity. After absorption, DOMGlcA can be formed followed by urinary excretion (Gratz et al., 2013). The incidence of DON and its glucuronides found in the BIOMYCO study are similar to the studies mentioned above, although the concentrations for the glucuronides are higher in Belgium.

Munoz et al. (2010) developed a method that allows analysis of **OTA** (LOQ-LOQ 0.02-0.05 ng/mL) and its detoxication product OTα (LOQ-LOQ 0.02-0.05 ng/mL) in urine. Application of the validated method in a pilot study with 13 volunteers from Germany revealed the presence of OTA (0.02-0.13 ng/mL) and OTα (0.05-4.7 ng/mL) in all samples. Duarte et al. (2010) evaluated the exposure of the Portuguese population (n= 155) to OTA (LOQ 0.008 ng/mL) whereby 92.2 % of the urine samples were positive for OTA (mean 0.018 ng/mL). The OTA incidence in the BIOMYCO study was lower (35-51 %) than the incidence reported in these previous performed studies. No levels of OTα could be found in Belgian urine despite the low LOD of the used method.

Blaszkewicz et al. (2013) developed a sensitive method for the analysis of **CIT and HO-CIT** in human urine and applied the method in a small pilot study in Germany. CIT was present in 8/10 urines (0.05-0.2 ng/mL) and HO-CIT (LOD-LOQ 0.05-0.10 ng/mL) was detected in 5/10 of the urine samples (0.15-1.12 ng/mL). Ali et al. (2014) applied the same method whereby CIT and HO-CIT were detected in 82 % and 84 % of all urine samples, at concentrations ranging from 0.02 to 0.08 ng/mL for CIT, and 0.05 to 0.51 ng/mL for HO-CIT. In the BIOMYCO study, the amount of positive samples was lower for CIT (59 %) although the concentrations were much higher (up to 1.4 ng/mL). The incidence of HO-CIT in the BIOMYCO study is much lower (6 and 12 %) despite the fact that the BIOMYCO study has a lower LOD.

Aflatoxins, fumonsins and zearalenones (<1 %) could not be detected or quantified in urine samples from the Belgian population. This is in contrast with earlier performed studies. Solfrizzo et al. (2011a) developed a **LC-MS/MS** method for simultaneous determination of

AFM_1, OTA, DON, DOM-1, α-ZEL, β-ZEL and FB_1 in human urine. The developed method was further used in a survey with 52 volunteers from Southern Italy (Solfrizzo et al., 2014). The presence of ZEN + ZEL's, OTA, DON, FB_1 and AFM_1 was detected in 100 % (0.120 ng/mL + 0.176 ng/mL), 100 % (2.129 ng/mL), 96 % (67.36 ng/mL), 56 % (0.352 ng/mL) and 6 % (0.146 ng/mL) of the samples, respectively. The BIOMYCO method was earlier pilot-tested in 32 Belgian volunteers whereby urine samples contained the same mycotoxins as in the BIOMYCO study but no aflatoxins, fumonisines and zearalenones were found. Aflatoxins and fumonisins occur in hot and humid climates, which can be a reason why they do not occur in Belgian urine.

8.1.3 Risk assessment

The estimated intake for DON varies between 0.11 – 19.57 µg/kg BW/day for children and 0.03 – 10.08 µg/kg BW/day for adults. Of total exposed subjects 56-69 % of children and 16-39 % of the adults were estimated to exceed the TDI. The percentages exceeding the TDI for DON are much higher than previous estimations reported in Europe and could be due to the high consumption of different food products such as bread, beer and cornflakes. In a study performed in Austria dietary intake was estimated between 0.38 and 2.2 µg/kg BW/day whereby 33 % of these volunteers exceeded the TDI. Within the BIOMYCO study, DON15GlcA was quantified using the calibration curve of DON3GlcA without any correction for higher response. This could have led to an overestimation of the DON intake compared to the method whereby the concentration of DON15GlcA was corrected for the higher response by the factor 1.88 (Warth et al., 2012a). The calculations used in that study were based on daily urine production of 2 L instead of 1.5 L in our study. Sarkanj et al. (2013) analysed urine of 40 pregnant women in Croatia whereby the intake was estimated between 0.1 and 33.1 µg/kg BW/day. Also in that study a daily urine volume of 2 L was used. 48 % of these women exceeded the TDI. Turner et al. (2010b) analysed 6 urine samples of 35 adults in the UK whereby 17 % exceeded the TDI (0.008 – 1.244 µg/kg BW/day). Gratz et al. (2014) analysed 2 urine samples of 15 adults in the UK and estimated the intake between 0.026 – 0.918 µg/kg BW/day. Wallin et al. (2013) evaluated the exposure of DON in Sweden. The

DON intake varied between 0.002 en 5.448 µg/kg BW/day and only 1 % of the adults exceeded the TDI. Some of these performed studies (Turner et al., 2010b; Wallin et al., 2013) used the indirect method to measure DON-glucuronides. Interference of the used β-glucuronidase with mucopolysaccharides and an incomplete hydrolysis, could be two reasons why these studies have lower intakes than the BIOMYCO study. On the other hand, most of these studies were performed on a limited number of participants and a limited number of toxins. Rodriguez-Carrasco et al. (2014b) analysed urine samples of 6 children, 16 adolescents and 22 adults in Spain, but only studied DON and DOM-1 concentrations without hydrolysis. The DON intake was estimated between 0.06 and 1.07 µg/kg BW/day (8 % >TDI). Solfrizzo et al. (2014) analysed 52 urine samples of South-Italy with hydrolysis. When estimating the intake of DON, the assumption that 50 % of the ingested DON is excreted in urine was used instead of 72 % in the BIOMYCO study. The estimated mean intake was 5.90 µg/kg BW/day whereby 6 % of the adults exceeded the TDI. Differences in exposure could be explained to the different dietary habits between different countries and the higher occurrence of DON in temperate climates. All these different approaches to estimate the intake of DON make it difficult to compare the results between different countries and hence to know if the TDI is exceeded.

For OTA the individually urinary level was used to carry out an exposure assessment. The estimated intake for OTA was between 0.2 – 100 ng/kg BW/day for children and 0.1 – 21 ng/kg BW/day for adults. In total 1 % of the Belgian population exceeded the TDI for OTA. Solfrizzo et al. (2014) analysed 52 urine samples of South-Italy, whereby 94 % exceeded the TDI. The mean intake of OTA was calculated based on the assumption that 2.6 % of the ingested OTA is excreted in urine (Gambacorta et al., 2013) and was 0.139 µg/kg BW/day. The levels found in this study were higher than the levels found in the BIOMYCO project. This difference can be due to the fact that the consumption of wine and dried fruit is less in Belgium when comparing to South-European countries. Furthermore no OTA metabolites were found in the urine samples of the Belgian population, in contrast with other studies (Coronel et al., 2011; Munoz et al., 2010). This can have led to an underestimation of the OTA intake in Belgium.

The estimated total intake for DON and OTA could imply a health risk as 16 to 69 % of the cases exceeded the TDI for DON (depending on the approach applied to calculate the intake) and 1 % the TDI for OTA. It has to be highlighted that these calculations are based on assumptions (% urinary excretion) and that inter-individual variations were not taken into account. Due to the lack of toxicokinetic data on mycotoxins in human, there are still a lot of uncertainties that should be taken into account when perform a risk assessment based on urinary mycotoxin levels. Additionally, the presence of unknown metabolites can lead to an underestimation.

In general, in this kind of exposure assessments young children need special attention because of the quantitative higher food intake per kg body weight. In order to perform more accurate estimations, more research needs to be done in order to collect more information about the human metabolism of mycotoxins, especially for CIT. More exposure assessment studies are needed in order to compare the different estimates and to evaluate the variability amongst people.

8.1.4 Urinary biomarkers in relation to food consumption and socio-demographical characteristics

Within the BIOMYCO study, exposure of different subgroups to DON, OTA and CIT was compared and differences between the mycotoxin concentrations measured for consumers and non-consumers of 43 food items was estimated, to explore whether the mycotoxin exposure could be explained by the consumption of certain foods.

The urinary mycotoxin concentrations measured in the Belgian population differed significantly among age and gender. Additionally both negative and positive correlations were found between BMI and urinary mycotoxin levels in adults and children. A significant difference could be seen for diets, whereby children with only a West-European diet had higher levels of creatinine-adjusted DON in their urine in comparison with children who

followed a combination of different diets. A significant difference could be seen for pregnant women whereby the levels of CIT-equivalents were lower in comparison with non-pregnant women. Also the urinary levels of creatinine-adjusted OTA were lower for pregnant women. Additionally, no significant difference in exposure between smokers and non-smokers could be seen.

Only a few studies investigated the differences of mycotoxin exposure between different subgroups on the one hand and the relation between urinary biomarkers and food consumption on the other hand. Ezekiel et al. (2014) conducted a study in Nigeria whereby AFB_1 and FB_1 levels in the food were significantly correlated with the excreted AFM_1 and FB_1 respectively. Family exposure patterns based on urinary concentrations were observed for AFB_1, FB_1, OTA and ZEN (based on ZEN14GlcA). A study conducted in Spain showed differences in OTA urine levels at different stages of breastfeeding (Munoz et al., 2014) whereby higher OTA levels were found in infant urines collected a few days after delivery compared to samples collected from infants who consumed mature milk. In the same study a relationship between OTA intake with breast milk and OTA levels in urine of the infants was observed. Furthermore Sarkanj et al. (2013) performed a biomarker study in Croatia in order to identify trends in mycotoxin exposure of pregnant women. The analyses revealed an insignificantly higher exposure to DON of the rural subgroup compared to the urban subgroup. Due to the low sample number there were no statistically significant correlations found between food intake and urinary DON equivalents, BMI or age. Abia et al. (2013) investigated the difference in exposure between HIV negative and positive populations in Cameroon. Overall there was no significant difference between the mean concentrations of analytes among the subgroups. Despite all these studies a lot of questions still remain to understand which factors influence the exposure to mycotoxins and which subpopulations are at higher risk.

In order to investigate the link between the presence of mycotoxins in urine and food consumption data, the population was divided in two classes for each food commodity (consumers and non-consumers). In a next step, the mycotoxin concentrations were

compared between the different classes whereby the consumption of 21 food items were related to higher/lower levels of OTA, DON, CIT, DON- and CIT-equivalents. The relationship between food consumption and urinary biomarker levels was earlier examined in the UK (Turner et al., 2010b). Within this study DON was assessed in first morning urine collected during a period of normal diet, a wheat-restriction intervention diet and partial wheat-restriction intervention in which bread was allowed. A strong correlation was found between wheat intake and urinary DON. In another study performed in the UK (Turner et al., 2008c) cereal intake was significantly associated with urinary DON. In multivariable analysis wholemeal bread, white bread, other bread, buns/cakes, high-fiber breakfast cereal and pasta were significantly associated with urinary DON. In a study performed in Spain (Coronel et al., 2011) a correlation was found for OTA and OTα in urine and the consumption of possible contaminated food commodities. Ezekiel et al. (2014) conducted a study in Nigeria whereby AFB_1 and FB_1 levels in the food were significantly correlated with the excreted AFM_1 and FB_1 respectively. Furthermore Sarkanj et al. (2013) performed a biomarker study in Croatia where no statistically significant correlations found between food intake due to the small sample size. Additionally, urinary DON was associated with intake of total cereal grain as well as whole grain, breakfast cereals and porridge consumption in Sweden (Wallin et al., 2013).

Some of the significant differences found in the BIOMYCO study could be explained by the earlier reported correlations with occurrences of mycotoxins in food commodities. On the other hand, these results raise questions regarding other dietary sources which contributed significantly to the urinary levels in the Belgian population. Any conclusion drawn at this phase of the research might be too preliminary. A ‘duplicate diet study’ whereby mycotoxins would be analysed in food consumed by a group of participants combined with a (24hours)-urine collection should be ideal to further investigate the link found between urinary biomarkers and food consumption data.

8.2 STRENGTHS AND LIMITATIONS

The major strength of this study was the large sample size and the representative distribution between age and gender across the study group to assess possible differences between mycotoxin exposure. This is further strengthened by including all the different classes of mycotoxins and a large amount of their metabolites with special attention to the glucuronides, the major urinary detoxification route in humans for DON. Furthermore, this study included young children aged from 3 until 12 years old which is of interest because of their quantitative higher food intake per kg body weight. This is further strengthened by collecting data on the consumption of 43 foods in order to get an idea about the consumption of several food items of interest. Furthermore the consumption of both the previous day and month before the urine collection was asked in order to get information about the relation to biomarkers with a short and long half-life. Finally, the BIOMYCO study is the first biomarker study performed in Belgium what is strengthened by the fact that for the first time a standardized study protocol was used and described.

A first weakness of this study is the collection of only one urine sample per participant. Collecting one urine sample is useful to study the exposure at population level but does not give enough detailed information when studying the exposure at individual level. Collecting more urine samples per participant was not realistic as it was difficult to find people who wanted to participate. Additionally, collecting more samples was not feasible given the timeline and budget available in the BIOMYCO project. Second, no reference standards for the glucuronides were available and the quantification of DON15GlcA was based on the concentration of DON3GlcA, because of the small amount of standard available. Third, the inaccuracy of the reported food consumption as well as the limited list of food items included in the questionnaire could imply that not all important sources of mycotoxins in Belgium were identified. Fourth, the self-reported BMI could imply inaccurate results. Fifth, due to logistic and budget constraints we could not include children below 3 years old, teenagers between 13 and 18 years and elderly above 65 years. It is of interest to study the exposure to these subpopulations in the future. Finally, it has to be highlighted that it was

assumed that DON and CIT are excreted within 24 hours and OTA within 1 month after consumption when studying the link with food consumption. Next, the conversions of urinary concentrations to intake estimates were based on excretion rate assumptions and inter-individual variations were not taken into account. Still a lot of toxicokinetic data about mycotoxins in humans is needed in order to circumvent the uncertainty of this type of analysis. A 'duplicate diet study' or an intervention study whereby mycotoxins would be analysed in the food and (24hours)-urine could be a better approach to study the relation between food consumption and mycotoxin exposure.

CHAPTER 9

RECOMMENDATIONS FOR FUTURE RESEARCH

Mycotoxins have already been known for many centuries, still a lot of research questions remain. Thanks to the evolution of technologies in sampling and mycotoxin analysis, more and more information is revealed about mycotoxin identification, occurrence, toxicology and their related risk for human and animal health.

Sampling strategies

From the available literature and information obtained during the BIOMYCO study it became clear that there is really need for alternative sample strategies. In most of the exposure assessment studies, mycotoxin biomarkers are analysed in morning- or 24-hours urine. While random spot samples are simple to collect, they are rarely equivalent to 24-hour collections due to analyte variability throughout the day. On the other hand, 24-hour collections are inconvenient and study participants tend to make collection and reporting mistakes, such as missed samples and errors in measuring total urine volume, which can affect results. Performing these sampling techniques on a large-scale becomes very difficult since both methods are subject to collection and transportation issues.

A simple solution to eliminate most of the problems associated with liquid urine collections is to collect spot urine samples on filter paper. Dried urine sampling has been successfully applied for the determination of a wide range of compounds in different fields, such as toxicology and therapeutic drug monitoring (Barcenas et al., 2014; Lee et al., 2013) and could be useful in mycotoxin analysis where it is not yet available. Spot collections require no collection jug or refrigeration and improve sample transport and size. In developing

countries where most of the time no freezers are available, this sampling technique could be very useful. Urine dried on filter paper produces results nearly identical to liquid urine and several compounds and metabolites showed enhanced stability in dried urine spots compared with liquid samples as earlier reported (Carreno Balcazar and Meesters, 2014). Finally creatinine can also be measured in dried urine, providing a way to normalise results by using a correction factor taking into account hydration status (Antunes et al., 2013).

Analytical methods

The need for multi-mycotoxin analyses in biological matrices is constantly rising and the technology of choice is LC-MS/MS. A major challenge in mycotoxin biomarker research is the extremely low analyte concentrations present in biological fluids. Appropriate sample preparation protocols are crucial to obtain an acceptable sensitivity. However, polar conjugates such as glucuronides are frequently lost during common cleanup approaches. The available literature and the information obtained during the BIOMYCO study indicate that there is a lot of variability between the different methods regarding sensitivity and reported urinary biomarker levels. An important quality control measure is the use of certified reference materials. However, for mycotoxin biomarkers, there is no matrix reference material available that would make it possible to assess the measurement performance in the analysis of biologically important matrices such as human urine. Furthermore, the organisation of a proficiency test and the development of a standard method for the detection and quantification of mycotoxin biomarkers in urine samples will contribute to more accurate biomarker analysis.

In the past, most biomarker methods focused on parent mycotoxins rather than on conjugated forms as no (certified) calibrants are commercially available for these metabolites. Furthermore, the targeted LC-MS/MS methods only analyse known mycotoxins and metabolites. In comparison with quadrupole instruments, the use of high resolution mass spectrometry such as time-of-flight increases the measured mass accuracy and resolution. This dominant tool for structural characterisation offers new opportunities in

mycotoxin research such as untargeted and metabolomic studies in order to reveal new metabolization patterns of mycotoxins in biological fluids.

Mycotoxin risk assessment

In order to study the impact of mycotoxin exposure on public health, it is important to assess the risk related to mycotoxin exposure. Currently, human exposure assessment to these toxins is often based on calculations combining mycotoxin occurrence data in food with population data on food consumption. In order to improve and refine risk assessments, human biomonitoring has become an added value in evaluating exposure to mycotoxins. The direct measurement of biomarkers of exposure is the only available tool that integrates exposures from all sources. Biomarkers of the most common mycotoxins have been validated in biological fluids such as urine. The individual variation in ADME processes is integrated when using biomarkers, whereby a more accurate assessment of exposure can be performed at the individual level. Furthermore, human biomonitoring can demonstrate trends and changes in exposure, establish distribution of exposure, identify vulnerable groups and it can reduce the assumptions regarding consumption rates. For this reason, the combination of exposure data from both methods will lead to a better interpretation in risk assessment (Choi et al., 2015).

The major limitations of human biomonitoring are the lack of standardisation in methodology and the fact that human biomonitoring alone cannot provide information about the source of exposure. Comparison between different performed studies is difficult due to various factors such as differences in age, differences in detection limits, limited numbers of subjects in other studies and different analytical performances of the methods. A standardised study protocol should be implemented in order to compare the exposure of different populations.

Further efforts have to be taken to increase usability of human biomonitoring. In order to translate human biomonitoring data into daily exposure estimates there is need for detailed understanding of the toxicokinetics. Within the BIOMYCO study a risk assessment was performed by comparing the estimated dietary intake of DON and OTA with their TDI. The dietary intake of DON and OTA was estimated using the urinary concentrations. It has to be highlighted that the used calculations were based on a lot of assumptions and that inter-individual variations in ADME processes were not taken into account. Due to the lack of information about the urinary mycotoxin excretion, no risk assessment could be performed for some of the mycotoxins. Still a lot of toxicokinetic and toxicological data about mycotoxins in humans are needed in order to circumvent the uncertainty of the statistical analysis, especially for CIT.

Findings on the high prevalence of CIT and OTA observed in the BIOMYCO study highlight the need for more research concerning the exposure to these two toxins with focus on identifying other possible sources of mycotoxin exposure. Since fungal species are able to produce more than one mycotoxin, people are exposed to mixtures of mycotoxins. The additive, synergetic or antagonistic effects of mycotoxins and their influences on human health effects are still not well known and need to be further investigated.

Some of the significant differences between consumers and non-consumers of different food commodities found in the BIOMYCO study could be explained by the earlier reported occurrences of mycotoxins in food commodities. On the other hand, the results raise questions regarding other dietary sources which contributed significantly to the urinary levels in the Belgian population. Any conclusion drawn at this phase of the research might be too preliminary. A ‘duplicate diet study’ whereby mycotoxins are analysed in food consumed by a group of participants or an intervention study whereby people need to follow a restricted diet, both combined with a (24hours)-urine collection could reveal more information to help with these questions.

Still a lot of questions remain to understand which factors influence the exposure to mycotoxins and which subpopulations are at higher risk. In general, further research should focus on exposure assessments in different subpopulations such as young children because of their relative high dietary intake per kg body weight or pregnant women because of the potential transfer of mycotoxins to the foetus.

Finally, the determination of the variability of mycotoxin exposure amongst people using standardised study protocols and the analysis of the related uncertainties, could help policy makers to establish health based guidance values for mycotoxins in urine. Future research revealing more information about mycotoxin identification, occurrence, toxicology and their related risk could help risk managers to set recommendations for prevention and control of mycotoxins.

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Summary

SUMMARY

The manuscript entitled '*Human biomonitoring of mycotoxin exposure through biomarker analysis*' describes the results of the BIOMYCO study whereby an exposure assessment of the Belgian population to mycotoxins was performed using urinary biomarkers of exposure.

Mycotoxins are secondary metabolites produced by fungi. These naturally occurring toxins are important harmful food contaminants responsible for different toxicological effects. In the general introduction an overview of the main characteristics of aflatoxins, fumonisins, trichothecenes, ochratoxins, zearalenone (ZEN) and citrinin (CIT) are presented (**Chapter 1**). In **Chapter 2**, a detailed description is given about the toxicokinetics and the main toxicological effects of these toxins. Next, the formation of mycotoxin biomarkers, the existing analytical methods to measure mycotoxin biomarkers in urine and the available biomonitoring studies earlier performed were summarised (**Chapter 3**).

In order to study the impact of mycotoxins on the public health, it is important to assess human exposure. Currently, human exposure assessment to these toxins is often based on calculations combining mycotoxin occurrence data in food with population data on food consumption. Because of uncertainties inherent to that approach, human biomonitoring has become an added value in evaluating exposure to mycotoxins in order to improve and refine risk assessments. The direct measurement of biomarkers of exposure is the only available tool that integrates exposures from all sources. Biomarkers of the most common mycotoxins have been validated in biological fluids such as urine. The individual variation in ADME processes is integrated when using biomarkers, whereby a more accurate assessment of exposure can be performed at the individual level. For this reason, the BIOMYCO study was designed to assess human mycotoxin exposure in Belgium using urinary biomarkers of exposure (**Chapter 4**).

Morning urine was gathered in a representative part of the Belgian population according to a designed study protocol, whereby 155 children (3-12 years old) and 239 adults (19-65 years old) were selected based on random cluster sampling. Every participant completed a food frequency questionnaire to assess the consumption of relevant foodstuffs (n= 43) of both the day and month before the urine collection. The study protocol was approved by the ethical committee of Ghent University Hospital and is described in **Chapter 5**.

A total of 394 urine samples were analysed for the presence of 33 potential biomarkers with focus on aflatoxins, CIT, fumonisins, trichothecenes, ochratoxin A (OTA), ZEN and their metabolites using two validated liquid chromatography tandem mass spectrometry (LC-MS/MS) methods. Nine out of the 33 analysed mycotoxins were detected whereby deoxynivalenol (DON), OTA, CIT and their metabolites deoxynivalenol-3-glucuronide (DON3GlcA), deoxynivalenol-15-glucuronide (DON15GlcA), deepoxy-deoxynivalenol-glucuronide (DOMGlcA) and dihydrocitrinone (HO-CIT) were the most frequently detected. DON15GlcA was the main urinary DON biomarker found and for the first time DOMGlcA was detected in children's urine. Furthermore α-zearalenol and β-zearalenol-14-glucuronide were found in respectively one and two samples. Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, aflatoxin M_1, fumonisin B_1, fumonisin B_2, fumonisin B_3, hydrolysed fumonisin B_1, diacetoxyscirpenol, 3-acetyldeoxynivalenol, 3-acetyldeoxynivalenol-15-glucuronide, 15-acetyldeoxynivalenol, 15-acetyldeoxynivalenol-3-glucuronide, deepoxy-deoxynivalenol, fusarenon X, HT-2 toxin, ochratoxin α, T-2 toxin, ZEN, zearalenone-14-glucuronide, α-zearalenol-7-glucuronide, α-zearalenol-14-glucuronide and β-zearalenol could not be detected or quantified in urine samples from the Belgian population. Additionally, other still unknown mycotoxin metabolites could be present in urine. Based on the urinary levels, the daily intake of DON and OTA was estimated and evaluated whereby, depending on the used method, 16-69 % of the population exceeded the tolerable daily intake for DON and 1 % for OTA (**Chapter 6**). These estimations, however, were based on a number of assumptions, and the results should therefore be interpreted with caution.

Exposure to DON, OTA and CIT was compared between subgroups (age, gender, …) whereby urinary mycotoxin concentrations measured in the Belgian population differed significantly among age and gender. Additionally a negative correlation was found between body mass index (BMI) and creatinine-adjusted levels of DON3GlcA and DON15GlcA and a positive correlation was found between BMI and creatinine-adjusted HO-CIT in adults. Furthermore a negative correlation was found between BMI and CIT in children using a Spearman correlation analysis. No significant difference in exposure between smokers and non-smokers could be seen. Finally, a link between the mycotoxin concentrations measured and the food consumption reported was estimated, to explore whether the mycotoxin exposure could be explained by the consumption of certain foods (**Chapter 7**). Statistical analysis of food consumption data, revealed a link between urinary levels of DON, OTA and CIT and the consumption of some food commodities (21/43). However, it has to be remarked that these trends should be confirmed by future research.

The BIOMYCO study is the first study whereby a multi-toxin approach was applied for mycotoxin exposure assessment in adults and children on a large-scale. Moreover, it is the first study that described the exposure to an elaborated set of mycotoxins in the Belgian population. Biomarker analysis showed a clear exposure of a broad segment of the Belgian population to DON, OTA and CIT. The risk assessment based on these data indicate a potential concern for a number of individuals whereby young children need special attention because of the relatively higher food intake per kg body weight (**Chapter 8**). The knowledge gathered through this study gives rise to new research questions and the need for future research (**Chapter 9**).

Samenvatting

SAMENVATTING

Het doctoraatsproefschrift getiteld '*Human biomonitoring of mycotoxin exposure through biomarker analysis*' beschrijft de resultaten van de BIOMYCO studie waarbij de blootstelling van de Belgische bevolking aan mycotoxines in kaart werd gebracht door gebruik te maken van blootstellingsbiomerkers in urine.

Mycotoxines zijn secundaire metabolieten die geproduceerd worden door schimmels. Het zijn natuurlijk voorkomende toxines die een belangrijke chronische risicofactor vormen in onze voedselketen en die verantwoordelijk zijn voor verschillende toxische effecten. In de algemene inleiding wordt een overzicht gegeven van de belangrijkste eigenschappen van aflatoxines, fumonisines, trichothecenen, ochratoxines, zearalenone (ZEN) en citrinine (CIT) **(Hoofdstuk 1)**. In **Hoofdstuk 2** wordt een gedetailleerde beschrijving gegeven over de toxicokinetiek en de belangrijkste toxicologische effecten van deze toxines waarna in **Hoofdstuk 3** de vorming van mycotoxine biomerkers wordt samengevat. Tenslotte wordt een overzicht gegeven van de bestaande analytische methodes voor de bepaling van mycotoxine biomerkers in urine als ook van de beschikbare blootstellingstudies die eerder werden uitgevoerd.

Om de mogelijke impact van mycotoxines op de volksgezondheid te bestuderen, is het noodzakelijk om de blootstelling van de bevolking aan deze toxines na te gaan. Blootstellingstudies zijn tot op heden gebaseerd op innameberekening op basis van concentraties van mycotoxines in voedingsmiddelen in combinatie met voedselconsumptiegegevens. Deze indirecte benadering heeft echter enkele onzekerheden, waardoor humane biomonitoring een meerwaarde is geworden bij het evalueren van de blootstelling. De directe meting van blootstellingsbiomerkers is de enige tool die blootstelling via verschillende routes integreert. Verder, kan er via de directe meting van blootstellingsbiomerkers in urine informatie verkregen worden over de individuele blootstelling aan mycotoxines. Een blootstellingstudie van de Belgische bevolking aan

mycotoxines via biomerkers was tot voor de start van dit project nog niet beschikbaar **(Hoofdstuk 4)**.

Ochtendurine van de Belgische bevolking werd verzameld volgens een vooraf opgesteld studieprotocol waaraan 155 kinderen (3-12 jaar) en 239 volwassenen (19-65 jaar) hebben deelgenomen. De nodige deelnemers werden door middel van clustersampling gerekruteerd. Alle deelnemers vulden ook een voedselfrequentievragenlijst in die peilde naar de inname van bepaalde relevante voedingsmiddelen (n=43) en de hoeveelheid ervan, zowel voor 24u als de maand voorafgaand aan de urinecollectie. Het studieprotocol werd goedgekeurd door het Ethisch Comité van het Universitair Ziekenhuis te Gent en wordt beschreven in **hoofdstuk 5**.

In totaal werden 394 urinestalen geanalyseerd op de aanwezigheid van 33 potentiële biomerkers met focus op aflatoxines, CIT, fumonisines, trichothecenen, ochratoxine A (OTA), ZEN en hun metabolieten door gebruik te maken van twee gevalideerde vloeistofchromatografie tandem massaspectrometrische methoden (LC-MS/MS). Negen van de 33 mycotoxines werden gedetecteerd in de urine van de Belgische bevolking waarbij deoxynivalenol (DON), OTA, CIT en hun metabolieten deoxynivalenol-3-glucuronide (DON3GlcA), deoxynivalenol-15-glucuronide (DON15GlcA), deepoxy-deoxynivalenol-glucuronide (DOMGlcA) en dihydrocitrinone (HO-CIT) het meest frequent werden teruggevonden. DON15GlcA bleek de belangrijkste urinaire DON metaboliet te zijn en werd in 100 % van de stalen teruggevonden. Voor de eerste keer werd ook het DOMGlcA gedetecteerd in de urine van kinderen. Verder werden α-zearalenol en β-zearalenol-14-glucuronide teruggevonden in respectievelijk één en twee stalen. Aflatoxine B_1, aflatoxine B_2, aflatoxine G_1, aflatoxine G_2, aflatoxine M_1, fumonisine B_1, fumonisine B_2, fumonisine B_3, gehydrolyseerd fumonisine B_1, diacetoxyscirpenol, 3-acetyldeoxynivalenol, 3-acetyldeoxynivalenol-15-glucuronide, 15-acetyldeoxynivalenol, 15-acetyldeoxynivalenol-3-glucuronide, deepoxy-deoxynivalenol, fusarenon X, HT-2 toxine, ochratoxine α, T-2 toxine, ZEN, zearalenone-14-glucuronide, α-zearalenol-7-glucuronide, α-zearalenol-14-glucuronide en β-zearalenol werden niet gedetecteerd in de urine van de Belgische bevolking. De

mogelijkheid bestaat echter dat er andere nog onbekende mycotoxine metabolieten aanwezig zijn in urine. Op basis van de urinaire concentraties van DON en OTA werd de blootstelling via voedselinname ingeschat. Deze blootstelling werd geëvalueerd waarbij, afhankelijk van de gebruikte methode, 16 tot 69 % van de Belgische bevolking de tolereerbare dagelijkse inname overschreed voor DON en 1 % voor OTA **(Hoofdstuk 6)**. Deze innameschattingen zijn echter gebaseerd op een aantal veronderstellingen, waardoor deze resultaten met enige voorzichtigheid moeten geïnterpreteerd worden.

De blootstelling aan DON, OTA en CIT werd vergeleken tussen verschillende subgroepen (leeftijd, geslacht, …) waarbij onder andere een significant verschil gevonden werd in urinaire mycotoxine concentraties naar gelang de leeftijd en het geslacht bij volwassenen en/of kinderen. Bijkomend werd een negatieve significante correlatie gevonden tussen de body mass index (BMI) en met creatinine-gecorrigeerde concentraties van DON3GlcA en DON15GlcA als ook een positieve significante correlatie tussen BMI en met creatinine-gecorrigeerde concentraties van HO-CIT in volwassenen. Verder werd ook een negatieve significante correlatie gevonden tussen BMI en CIT in kinderen gebruik makend van de Spearman correlatie-analyse. Er werd geen significant verschil waargenomen tussen rokers en niet-rokers. Tenslotte werd er een verband tussen de gemeten mycotoxine concentraties in urine en de gerapporteerde consumptie van verschillende voedingsmiddelengroepen onderzocht om na te gaan of de blootstelling aan mycotoxines kon verklaard worden door de consumptie van bepaalde voedingsmiddelen **(Hoofdstuk 7)**. Na de statistische analyse van de voedselconsumptiegegevens, kon een verband gezien worden tussen de urinaire concentraties van DON, OTA en CIT en de consumptie van enkele voedingsmiddelen (21/43). Deze resultaten moeten echter gezien worden als een eventuele trend die door verder onderzoek moet bevestigd worden.

De BIOMYCO studie is de eerste studie waarbij een multi-toxine benadering werd gebruikt bij blootstellingstudies via urinaire biomerkers van volwassenen en kinderen op grote schaal. Verder is het ook de eerste studie die de blootstelling van de Belgische bevolking aan mycotoxines via biomerkers beschrijft. De biomerkeranalyse toonde een duidelijke

blootstelling van de Belgische bevolking aan DON, OTA en CIT. De risico-analyse gebaseerd op deze resultaten duidt een potentieel risico aan voor een aantal individuen waarbij speciale aandacht moet gegeven worden aan jonge kinderen gezien hun relatief hogere voedselinname in vergelijk met hun lichaamsgewicht **(Hoofdstuk 8)**. De kennis die deze studie verzameld heeft, doet nieuwe onderzoeksvragen oprijzen en toont de nood voor verder onderzoek aan **(Hoofdstuk 9).**

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About the author

ABOUT THE AUTHOR

Ellen Heyndrickx was born in Ghent on January 31st, 1988. In September 2006, her university education was started at Ghent University (Belgium) and in 2011 she graduated as a master in Pharmaceutical Sciences. The master thesis 'Development of a multi-mycotoxin UPLC-MS/MS method for silages' was accomplished at the Institute of Agricultural and Fisheries Research (Melle, Belgium) under supervision of Prof. Sarah De Saeger and Dr. Els Daeseleire. Shortly after obtaining her master degree she was admitted to the Laboratory of Food Analysis (Faculty of Pharmaceutical Sciences, Ghent University) to start her PhD under promotership of Prof. Sarah De Saeger and Dr. Isabelle Sioen. The research received funding from the Belgian Federal Public Service of Health, Food Chain Safety and Environment (BIOMYCO RT11/02) and a scholarship from the Special Research Fund of Ghent University (BOF14DC1005) was obtained. Results of her research were published in a number of peer-reviewed scientific journals and were presented during several national and international conferences. During the PhD period, she combined her work with assisting 6 master thesis students. In July 2015, Ellen Heyndrickx finalised her PhD research and started a new career as a post-doctoral researcher at the Laboratory of Food Analysis where she will further contribute to mycotoxin biomarker research within the European FoodBAll project (JPI-BioNH-FWO).

A1 PEER REVIEWED PUBLICATIONS

Assessment of mycotoxin exposure in the Belgian population using biomarkers: Aim, design and methods of the BIOMYCO study (2014) Ellen Heyndrickx, Isabelle Sioen, Mia Bellemans, Mieke De Maeyer, Alfons Callebaut, Stefaan De Henauw and Sarah De Saeger. *Food Additives & Contaminants: Part A, 2014 Vol. 31, No. 5, 924–931.*

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FOLLOWED COURSES

Doctoral Schools in Life Sciences and Medicine (2011-2015) at Ghent University, Belgium

Pipetting and Calibration (2012) at VWR, Leuven, Belgium

Short Training Initiative: Intensive training on mycotoxin analysis (2012) at Ghent University, Belgium

Design of experiments (2012) at Ghent University, Belgium

Multivariate analysis (2012) at Ghent University, Belgium

Training in Metrology in Chemistry (2012) organised by European Commission at Ghent University, Belgium

Training LC-MS instrument XEVO TQ-S (2014) organised by Waters at Ghent University, Belgium

Quality management in laboratories (2014) at Ghent University, Belgium

Project Management (2014) organised by T.O.M Bvba at Ghent University, Belgium

Advanced Academic English Effective Slide Design (2015) at Ghent University, Belgium

Leadership Foundation course (2015) organised by Dan Steer at Ghent University, Belgium

Training HR-MS instrument Synapt G2-S (2015) organised by Waters at Ghent University, Belgium

TUTORSHIP MASTER THESES

Stefanie De Deurwaarder (2011-2012) Blootstellingstudie van de Belgische bevolking aan mycotoxines: Ontwikkelen van een (UP)LC-MS/MS methode voor de bepaling van mycotoxine biomerkers in urine. *Faculty of Pharmaceutical Sciences, Ghent University, Belgium.*

Lonis Van Belleghem (2012-2013) Blootstellingstudie van de Belgische bevolking aan mycotoxines via biomerkers: Lente 2013. *Faculty of Pharmaceutical Sciences, Ghent University, Belgium.*

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Abbreviations

ABBREVIATIONS

3ADON	3-acetyldeoxynivalenol
3ADON15GlcA	3-acetyldeoxynivalenol-15-glucuronide
4-OH-OTA	4-hydroxy-ochratoxin A
10-OH-OTA	10-hydroxy-ochratoxin A
15ADON	15-acetyldeoxynivalenol
15ADON3GlcA	15-acetyldeoxynivalenol-3-glucuronide
α-ZAL	α-Zearalanol
α-ZEL	α-Zearalenol
α-ZEL7GlcA	α-Zearalenol-7-glucuronide
α-ZEL14GlcA	α-Zearalenol-14-glucuronide
ADME	Absorption, distribution, metabolism and excretion
AFAR	Aflatoxin aldehyde reductase
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFB_{2a}	Aflatoxin B_{2a}
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFL	Aflatoxicol
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFQ_1	Aflatoxin Q_1

ATA	Alimentary toxic aleukia
ATP	Adenosine triphosphate
β-ZAL	β-Zearalanol
β-ZEL	β-Zearalenol
β-ZEL14GlcA	β-Zearalenol-14-glucuronide
BMI	Body mass index
BW	Body weight
CEN	European Standardisation Committee
CIT	Citrinin
CF	Concentration factor
CRL	Certified reference laboratory
CYP	Cytochroom P450 enzymes
DAS	Diacetoxyscirpenol
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DON3Glc	Deoxynivalenol-3-glucoside
DON3GlcA	Deoxynivalenol-3-glucuronide
DON15GlcA	Deoxynivalenol-15-glucuronide
DOM-1	Deepoxy-deoxynivalenol
DOMGlcA	Deepoxy-deoxynivalenol-glucuronide
EC	European Commission
EFSA	European Food Safety Authorisation
ELEM	Equine leukeencephalomalacia

ER	Excretion rate
ESI	Electrospray ionisation
EU	European Union
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FFQ	Food frequency questionnaire
FusX	Fusarenon X
GC-MS/MS	Gas chromatography tandem mass spectrometry
GST	Glutathione S-transferase
HFB_1	Hydrolysed fumonisin B_1
HO-CIT	Dihydrocitrinone
HPLC-FD	High performance liquid chromatography with fluorescence detection
HT-2	HT-2 toxin
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
i.d.	Internal diameter
ILSI	International Life Science Institute
IPCS	International Programme on Chemical Safety
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MAPK	Mitogen activated protein kinases

MW	Molecular weight
NIV	Nivalenol
NTD	Neural tube defects
NOAEL	No observed adverse effect level
OTA	Ochratoxin A
OTα	Ochratoxin α
OTB	Ochratoxin B
OTC	Ochratoxin C
RASSF	Rapid Alert System for Food and Feed
RNA	Ribonucleic acid
ROS	Reactive oxidative species
Sa	Sphinganine
SCOOP	Scientific Cooperation on Questions relating to Food
SL	Sphingolipids
So	Sphingosine
SPE	Solid phase extraction
T-2	T-2 toxin
TDI	Tolerable daily intake
TWI	Tolerable weekly intake
UV	Ultraviolet
v/v	Volume to volume
WHO	World Health Organisation
ZAN	Zearalanone

ZEN	Zearalenone
ZEN14Glc	Zearalenone-14-glucoside
ZEN14GlcA	Zearalenone-14-glucuronide
ZEN14S	Zearalenone-14-sulphate

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SOCIO-DEMOGRAFISCHE GEGEVENS VOLWASSENEN

ID-nummer

In welk jaar werd u geboren?

Wat is uw geslacht ?

O Man O Vrouw

Wat is de postcode van uw huidige woonplaats?

Wat is uw lengte (cm)?

Wat is uw gewicht (kg)?

Heeft u een ziekte of aandoening met betrekking tot ...?

(U kan meerdere antwoorden aanduiden. Indien u geen gezondheidsproblemen heeft, dan kan u doorgaan naar de volgende vraag.)

O Lever O Gal O Nieren O Andere ----------

Welke geneesmiddelen heeft u de VOORBIJE MAAND ingenomen? Welke dosis?

(Indien u geen geneesmiddelen neemt, dan kan u naar de volgende vraag doorgaan.)

Geneesmiddel	Dosis	Geneesmiddel	Dosis

Bent u zwanger of bestaat er een kans dat u zwanger bent?

O Ja O Neen

Geeft u borstvoeding?

O Ja O Neen

Rookt u?

O Ja O Neen

Volgt u momenteel een dieet waarbij bepaalde voedingsmiddelen niet mogen gegeten worden dit omwille van ziekte, allergie of zonder enige reden?

(U kan meerdere antwoorden aanduiden.)

O Neen O Ja, namelijk

O Glutenvrij
O Lactose-beperkt
O Eiwit-beperkt
O Energiebeperkt (vermagering)
O Vet-/cholesterolarm
O Zoutarm (Bijvoorbeeld bij hoge bloeddruk, ...)
O Diabetes
O Andere ----------

Welk eetpatroon volgt u?

(U kan meerdere antwoorden aanduiden.)

O West-Europees
O Mediterraans
O Oosters
O Halal
O Kosjer
O Weinig vlees
O Vegetarisch
O Veganistisch
O Andere ----------

Hier niets
invullen a.u.b.

O ja

O neen

O geen informatie

Vragenlijst over Voeding - Afbraakproducten mycotoxines in urine

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UNIVERSITEIT GENT

federale overheidsdienst
VOLKSGEZONDHEID,
VEILIGHEID VAN DE VOEDSELKETEN
EN LEEFMILIEU

CODA - CERVA

Vragenlijst over Voeding - Afbraakproducten mycotoxines in urine

Deze vragenlijst werd opgesteld om de consumptie van een gelimiteerd aantal voedingsmiddelen na te gaan, en dit voor een welomschreven periode. De voedingsmiddelen opgelijst in deze vragenlijst omvatten dus slechts een klein deel van wat u mogelijk kan gegeten en gedronken hebben tijdens de voorbije maand/24u.
De bekomen gegevens zullen door de onderzoekers gebruikt worden om mogelijke verbanden te zoeken tussen de inname van deze voedingsmiddelen en bepaalde afbraakproducten van mycotoxines die voorkomen in de urine.

Deze vragenlijst wordt ingelezen met een scantoestel. Het is van groot belang dat het invullen als volgt wordt uitgevoerd:

- **Gelieve het bolletje van jouw keuze zwart te maken.** Gebruik hiervoor **geen viltstift** maar wel een **potlood** of balpen.
- **Indien gegeten/gedronken: maak het bolletje zwart dat overeenkomt met de totale hoeveelheid van 24u.**
- **Indien extra informatie gevraagd wordt: gelieve niet in de bolletjes te schrijven.**
- Indien u per ongeluk het verkeerde antwoord hebt ingekleurd dan kan u dat corrigeren door het te doorkruisen en vervolgens het juiste antwoord in te kleuren. Gelieve ook met een pijltje het juiste antwoord aan te duiden.

Bij het invullen van de vragenlijst is het heel belangrijk dat u rekening houdt met wat u gegeten/gedronken hebt gedurende de **maand** en **dag voorafgaand** aan de **ochtendurinecollectie**, ook al kan dit afwijken van uw normale eetpatroon.

- Voorbije maand = 4 weken voor de ochtenurinecollectie
- Voorbije dag = 24u voorafgaand aan de ochtendurinecollectie
- Voorbeeld - ochtendurinecollectie op maandag 1 oktober 2012:
  Voorbije maand = van 3 september 2012 tot 1 oktober 2012
  Voorbije 24u = van bij het opstaan op zondag 30 september 2012 tot net voor de ochtendurinecollectie van maandag 1 oktober 2012

Voor uw persoonlijk uitgewerkt voorbeeld: zie bijgevoegde instructiekaart

- Indien u de voedingsmiddelen tijdens de **voorbije maand** hebt gegeten/gedronken, gelieve dan in **kolom 3** een **gemiddelde hoeveelheid per dag** in te vullen en ga vervolgens naar kolom 4.
- Indien u de voedingsmiddelen ook tijdens de **voorbije 24u** hebt gegeten/gedronken, gelieve dan in **kolom 4** de **totale hoeveelheid** voor deze **24u** in te vullen.

Hebt u nog vragen, dan kan u ons altijd bereiken tijdens de kantooruren.
Mia Bellemans en Mieke De Maeyer: 09/332.36.78 - Ellen Heyndrickx: 09/264.81.33

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Van harte bedankt voor uw medewerking!

VOORBEELD: Ochtendurinecollectie op een maandag

De laatste 14 dagen voor de urinecollectie werd er dagelijks een brikje sojamelk gedronken.
De eerste 14 dagen van de voorbije maand (voorafgaand aan de urinecollectie) werd er geen sojamelk gedronken.
Tijdens de voorbije maand werden er elke ochtend, uitgezonderd op zondagen, 2 sneden roggebrood gegeten.
Enkel gedurende het laatste weekend, op zaterdag én zondag, werden er borrelnootjes gegeten: gemiddeld 1 eetlepel per dag.

1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
Sojadranken	○ nooit tijdens de voorbije maand ○ 1-3 dagen per maand ○ 1 dag per week ● 2-4 dagen per week ○ 5-6 dagen per week ○ elke dag	*indien gedronken, vul in:* tassen van 125 ml: ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* bekers/brikjes van 250 ml: ○ ● ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gedronken, vul in:* tassen van 125 ml: ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* bekers/brikjes van 250 ml: ○ ● ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1/2 1 2 3 4 5 6 7 8 9 10 +	
Roggebrood	○ nooit tijdens de voorbije maand ○ 1-3 dagen per maand ○ 1 dag per week ○ 2-4 dagen per week ● 5-6 dagen per week ○ elke dag	*indien gegeten, vul in:* sneden brood: ○ ● ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* sneden brood: ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1 2 3 4 5 6 7 8 9 10 11 12 +	
Pinda's, pindakaas *10 stuks gepelde pindanoten = 20 g* *1 eetlepel (borrel)nootjes = 20 g* *1 eetlepel pindakaas = 15 à 20 g*	○ nooit tijdens de voorbije maand ● 1-3 dagen per maand ○ 1 dag per week ○ 2-4 dagen per week ○ 5-6 dagen per week ○ elke dag	*indien gegeten, vul in:* eetlepels van 20 g: ● ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* eetlepels van 20 g: ● ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ ○ 1 2 3 4 5 6 7 8 9 10 11 12 +	

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Veel succes bij het invullen van de vragenlijst!

1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
DRANKEN				
Koffie *1 tas = 125 ml*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gedronken, vul in:* tassen van 125 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gedronken, vul in:* tassen van 125 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Sojadranken	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gedronken, vul in:* tassen van 125 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* bekers/brikjes van 250 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gedronken, vul in:* tassen van 125 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* bekers/brikjes van 250 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Bier *1 flesje/pilsglas = 250 ml* *1 fles zwaar bier = 330 ml* *1 blik = 330 of 500 ml*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gedronken, vul in:* glazen van 250 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* glazen van 330 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gedronken, vul in:* glazen van 250 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* glazen van 330 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
Wijn en **schuimwijn**	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gedronken, vul in:* glazen van 125 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gedronken, vul in:* glazen van 125 ml: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
ONTBIJTGRANEN				
Muesli en **cruesli** (krokante muesli) *1 kommetje muesli = 40 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* kommetjes van 40 g: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	*indien gegeten, vul in:* kommetjes van 40 g: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	O m m O m d O r m O r d O t m O t d O ? m O ? d
Ontbijtgranen (cornflakes, rice krispies, ...) *1 kommetje ontbijtgranen = 30g* *1 individuele verpakking = 20 à 30 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* kommetjes van 30 g: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	*indien gegeten, vul in:* kommetjes van 30 g: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	O m m O m d O r m O r d O t m O t d O ? m O ? d

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
BROOD				
Wit brood, pistolet, stokbrood *1 pistolet/piccolo = 40 à 45 g* *10 cm stokbrood = 40 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks pistolets/stokbrood (10 cm): O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks pistolets/stokbrood (10 cm): O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Roggebrood	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Bruin brood, granenbroden, volkoren brood, pistolet, stokbrood (alle "bruine" broodsoorten uitgezonderd roggebrood) *1 pistolet/piccolo = 40 à 45 g* *10 cm stokbrood = 40 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks pistolets/stokbrood (10 cm): O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks pistolets/stokbrood (10 cm): O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
Rozijnenbrood	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Fantasiebrood, sandwiches (suiker-, melkbrood, ...) *1 sandwich = 40 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks sandwiches, ...: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* sneden brood: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks sandwiches, ...: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Koffiekoeken (boterkoek, croissant, bolus, crèmekoek, ...)	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* stuks: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* stuks: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Beschuit, cracotte,knäckebröd, Zweedse broodjes	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* stuks: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* stuks: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
GEBAK EN KOEK		*indien gegeten, vul in:*	*indien gegeten, vul in:*	
Granenrepen (Special K-reep, mueslireep,...)	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	repen: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	repen: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	O m m O m d O r m O r d O t m O t d O ? m O ? d
Peperkoek	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* sneden van 23 g: : O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* sneden 23 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Koekjes (petit beurre, speculaas, vanillewafel, eierkoek, ...) *1 petit beurre/speculaas = 7 g* *1 sprits = 20 g* *1 prince koek = 22 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* koekjes van +/- 10 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* koekjes van +/- 10 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Pannenkoeken	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* pannenkoeken: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* pannenkoeken: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
Cake (madeleintje, gemarmerde cake, ...) *1 plakje cake = 30 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* plakjes/stuks cake: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* plakjes/stuks cake: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Gebak en **taart**	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* spies, individuele taartjes: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	*indien gegeten, vul in:* spies, individuele taartjes: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	
RIJST, DEEGWAREN en GRANEN				
"Ongepelde " rijst	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
"Witte/gepelde" rijst	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	

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Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
"Volkoren" deegwaren op basis van **tarwe**: spaghetti, macaroni, penne, ... *120g "droge" deegwaren geeft 300 g gekookte deegwaren.*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* borden van 300g bereid: (als hoofdschotel, 1 portie- volwassenen) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* borden van 150 g bereid: (als begeleidend gerecht, kleine portie) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* borden van 300g bereid: (als hoofdschotel, 1 portie- volwassenen) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* borden van 150 g bereid: (als begeleidend gerecht, kleine portie) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
"Niet volkoren" deegwaren op basis van **tarwe**: spaghetti, macaroni, penne, bulgur, couscous, Ebly granen, ...	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* borden van 300g bereid: (als hoofdschotel, 1 portie- volwassenen) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* borden van 150 g bereid: (als begeleidend gerecht, kleine portie) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* borden van 300g bereid: (als hoofdschotel, 1 portie- volwassenen) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* borden van 150 g bereid: (als begeleidend gerecht, kleine portie) O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
Deegwaren op basis van **maïs** (polenta, ...)	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* eetlepels bereid: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
PIZZA, PITA en TORTILLA (wrap)		*indien gegeten, vul in:*	*indien gegeten, vul in:*	
Pizza, pita (durum) *1mini pizza (aperitiefhapje) = 20 g* *1 kleine pizza (diam.13cm) = 150 g* *1 middelgr. pizza (diam.25cm) = 350 g* *1 pitabroodje (diam.15cm) =70 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	mini pizza van 20 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* kleine pizza van 150 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* spie van 60 g: (1/6 van een middelgrote pizza) O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* broodje/wrap van 70 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	mini pizza van 20 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* kleine pizza van 150 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* spie van 60 g: (1/6 van een middelgrote pizza) O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *en/of* broodje/wrap van 70 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Tortilla (wrap), op basis van **maïs** *1 tortilla (diam.20 cm) = 40 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* tortilla/wrap van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	*indien gegeten, vul in:* tortilla/wrap van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
SOJAPRODUCTEN		*indien gegeten, vul in:*	*indien gegeten, vul in:*	
Pudding, pap, desserten, ... op basis van **soja** *1 industrieel potje sojadessert = 125 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	potjes, kommetjes van 125 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	potjes, kommetjes van 125 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	
Tofoe	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	plakjes van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	plakjes van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Tempé	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	plakjes van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	plakjes van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Vegetarische burger op basis van **tofoe en/of tempé** (alle andere burgers NIET meerekenen)	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* burgers van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	*indien gegeten, vul in:* burgers van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
NOTEN en GEDROOGD FRUIT				
Studentenhaver	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels van 20 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* eetlepels van 20 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Pinda's, pindakaas *10 stuks gepelde pindanoten = 20 g* *1 eetlepel (borrel)nootjes = 20 g* *1 eetlepel pindakaas = 15 à 20 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels van 20 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* eetlepels van 20 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Pistachenoten *1eetlepel pistachenoten met dop = 20 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels (met dop) van 20 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* eetlepels (met dop) van 20 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Overige noten	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* handjes van 25 à 30 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* handjes van 25 à 30 g: O O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
Gedroogd fruit: **rozijnen** *1 eetlepel rozijnen= 20 g* *1 klein doosje rozijnen = 42 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels van 12 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* doosjes van 42 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	*indien gegeten, vul in:* eetlepels van 12 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* doosjes van 42 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	
Gedroogd fruit: overige *1 gedroogde pruim, abrikoos of dadel = 8 g* *1 gedroogde vijg = 20 g*	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* stuks van 8 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks van 20 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* stuks van 8 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* stuks van 20 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
DIVERSE				
Popcorn	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* handjes van 10 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	*indien gegeten, vul in:* handjes van 10 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Chips"afgeleiden", NIET op basis van **aardappelen** (Doritos, tortillachips, Bugles, Chipitos, ...)	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* klein zakje van 30 à 40 g: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	*indien gegeten, vul in:* klein zakje van 30 à 40 g: O O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 + *Noteer naam & merk:* ..... *Noteer het meest gegeten product. Indien meerdere producten in gelijke hoeveelheden gegeten werden, noteer dan alle namen (merken).*	O m m O m d O ? m O ? d O nvtm O nvtd
Maïs, vers-diepvries-blik	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	*indien gegeten, vul in:* eetlepels: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* kolven: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	*indien gegeten, vul in:* eetlepels: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 + *en/of* kolven: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 +	

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1	2	3	4	
Voedingsgroepen	**Hoe vaak** hebt u dit voedingsmiddel gegeten/gedronken tijdens de **voorbije maand**?	En hoeveel dan **gemiddeld per dag**?	Gedurende de voorbije **24u** voorafgaand aan de ochtendurinecollectie, hoeveel van de volgende voedingsmiddelen hebt u **gegeten/gedronken**?	Hier niets invullen a.u.b.
		indien gegeten, vul in:	*indien gegeten, vul in:*	
Seitan	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	plakjes van 80 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	plakjes van 80 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 40 g: O O O O O O O O O O O O 1/2 1 2 3 4 5 6 7 8 9 10 +	
Quorn	O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag	burgers van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 20 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	burgers van 75 g: O O O O O O O O O 1/4 1/2 1 2 3 4 5 6 + *en/of* eetlepels van 20 g: O O O O O O O O O O O O O 1 2 3 4 5 6 7 8 9 10 11 12 +	
Gedroogde specerij: paprika	O geen informatie O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag		Werd deze specerij gebruikt/gegeten tijdens de **voorbije 24u?** O ja O neen O geen informatie	
Gedroogde specerij: chili	O geen informatie O nooit tijdens de voorbije maand O 1-3 dagen per maand O 1 dag per week O 2-4 dagen per week O 5-6 dagen per week O elke dag		Werd deze specerij gebruikt/gegeten tijdens de **voorbije 24u?** O ja O neen O geen informatie	

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